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Award Number: DAMD17-00-C-0030

TITLE: Development and Production of a Leishmania Skin Test

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CONTRACTING ORGANIZATION: Allermed Laboratories, Inc.
San Diego, CA 92111

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14. ABSTRACT

Further refinement of the manufacturing process of Leishmania tropica Skin Test Antigen (LtSTA) was made during this contract period to increase the yield and robustness of the parasite during culture. Identity and potency tests for LtSTA were developed and the procedures are being validated. The analysis of dose-response, safety and efficacy data from a phase II clinical trial conducted in Sidi Bouzid, Tunisia in 2007 were compiled and analyzed, and a study report was completed and submitted to the U.S. Army and the FDA. The results of this investigation indicated that a 30ug dose could be used to determine the sensitivity and specificity of the product; the observed sensitivity of the 30ug dose was 0.85 and the observed specificity was 0.97. To evaluate the sensitizing properties of a 30ug does of LtSTA, a phase IIb study was planned and executed in August 2008. The trial was designed to determine if a 30ug dose could be administered intradermally three times without observing sensitization. The study also included 15ug and 50ug doses to evaluate the importance of product concentration in the induction of sensitivity. At the completion of this study, a Type B Meeting with the FDA will be requested to discuss the design of a phase III trial. Participants in this meeting will include Allermed personnel and members of the U.S. Army Leishmania interest group.

15. SUBJECT TERMS
LtSTA = Leishmania tropica Skin Test Antigen

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1. INTRODUCTION

Leishmaniasis is a common parasitic disease occurring throughout Africa, Asia, and Latin America.[1-4] The promastigotes (a form of the parasite found in the insect vector) is normally transmitted to humans by the bite of a female sand fly. Infection with *Leishmania* can result in a variety of clinical syndromes, conventionally divided into four major clinical groups: cutaneous, mucocutaneous, diffuse cutaneous, and visceral. The spectrum of disease is so wide that the result of an untreated infection with *Leishmania* ranges from asymptomatic to fatal disease.[5-7] Immunity is largely cell mediated; therefore, tests designed to detect cell-mediated immune responses in exposed individuals are more likely to represent true measures of infection. The use of a skin test to detect delayed-type hypersensitivity (DTH) is a convenient, simple and cost-effective method to assess cell-mediated immune response in humans and can be used for large-scale population surveys.[8] Although there are other methods of detecting cell-mediated immunity, these are difficult to perform, difficult to standardize, and are not practical for screening large numbers of individuals.The predictive values of DTH to *Leishmania* skin test antigen has been studied by numerous investigators, including the investigational team that conducted a phase II trial sponsored by Allermed.[9]

Leishmaniasis is a threat to soldiers deployed to endemic areas where the disease can cause significant morbidity in immunologically naive individuals. Reports from Iraq have raised concerns regarding the potential impact of cutaneous leishmaniasis on deployed personnel and disruption to unit readiness.[10-11] Past experience with this parasite has highlighted several areas of concern that a tuberculin-like skin test antigen of *Leishmania* could address. The antigen can be easily administered and may be used both diagnostically to confirm CL in military personnel with skin lesions and as a screening tool for asymptomatic undiagnosed cases in personnel returning from endemic areas.

2. BACKGROUND

Leishmania tropica Skin Test Antigen (LtSTA), is a sterile injectable microfluidized lysate of *Leishmania tropica* (WR#1063:C1A) promastigotes. The product is heat-treated, filtered, and formulated to a known protein concentration in a solution of 0.85% sodium chloride, 0.4% phenol, 0.01% Tween-80, and 1% glycerin contained in phosphate buffer. The antigen is manufactured in compliance with current Good Manufacturing Practices (cGMP) at Allermed Laboratories, San Diego, CA 92111

LtSTA is being developed as a screening tool to diagnose *Leishmania* infection in persons who reside in the United States and who travel to endemic regions of the world. This effort is important for both military personnel serving throughout the Middle East and U.S. civilians who travel and work in this region. The product was first studied by the U.S. Army following the 1991 Gulf War and has been under IND development at Allermed since 2000.

Two clinical studies sponsored by Allermed have been conducted with LtSTA. An ascending dose-response safety trial was conducted in 2004-2005 in 32 adult volunteers in which four cohorts consisting of eight subjects per cohort were skin tested with the antigen. The doses studied were 20, 40, 80, 120μg. No adverse reactions were observed at these dose levels. However, the 120μg dose caused an inflammatory response at the skin test site after two weeks in two individuals, which suggested that these subjects had responded with a delayed-type hypersensitivity reaction to residual antigen in the skin. The final study report of this phase I safety trial was sent to the FDA and HRPO on March 20, 2006.

A phase II clinical study was conducted in 2007 in Tunisia. A dose-response component of the trial involved testing persons infected with *Leishmania major* with ascending doses of LtSTA at concentrations of 10, 20, 40, and 80μg. Five adult volunteers with active cutaneous leishmaniasis were tested with each dose. All subjects (100%) reacted with a positive induration response to the antigen. A best-fit line was calculated from the dose-response data and a dose of 30μg was selected for use in evaluating the sensitivity and specificity of the antigen. Forty volunteers with a history of cutaneous leishmaniasis within the past 24 months (sensitivity cohort) and forty persons without a history of the disease (specificity cohort) were skin tested from the same endemic area (Sidi Bouzid, Tunisia). Thirty-four subjects in the sensitivity cohort were skin test positive to LtSTA (85%) with a mean induration response of 15.5 mm. Based on experience with other DTH antigens, a 15.5 mm reaction is an optimum induration response for these products. In the forty volunteers without a history of cutaneous leishmaniasis, thirty-nine subjects (97%) were skin test negative to the 30μg dose, indicating a high level of product specificity.

An amendment to the 2007 protocol was approved by the local IRB in Tunisia to obtain blood samples from the six skin test negative subjects for further study. The lymphocytes of these individuals were compared with those of subjects with a positive skin test to LtSTA by the lymphocyte proliferation assay using both *L. major* and *L. tropica* soluble antigen. The six persons who were skin test negative to the 30μg dose were retested with a 50 μg dose of LtSTA approximately 3 months later and were found to be skin test positive. This finding suggested that either the 30μg dose was too small to elicit a positive skin test in some individuals, or the 30μg dose was responsible for sensitizing the lymphocytes of these subjects which resulted in a positive skin test to the 50 μg dose.

Before beginning a phase III trial involving a population of subjects that will provide acceptable statistical power for a sensitivity/specificity study for either a 30μg or 50μg dose of LtSTA, it was deemed advisable to first test both doses for sensitizing properties. The indication of LtSTA as a skin test antigen for single or multiple use depends upon the ability or inability of the antigen to sensitize the lymphocytes of naïve individuals. To determine if sensitization is dose dependent, a 15μg dose was evaluated in addition to a 30μg and 50μg dose of LtSTA.

The purpose of the trial was three-fold as follows: (1) to evaluate the safety of 15μg, 30μg and 50μg/0.1mL doses of LtSTA in healthy adult volunteers with no known previous exposure to *Leishmania* parasites; (2) to provide information on the occurrence of false-positive skin tests to the initial dose of LtSTA in *Leishmania* naïve persons; and (3) determine the effect of previous skin tests with each dose of LtSTA on the outcome of repeat tests at 30 and 60 days.

3. BODY OF REPORT

3.1 Statement of Work

3.1.1 Complete analysis of the data obtained from phase II clinical trial conducted in 2007 in Sidi Bouzid, Tunisia.

3.1.2 Submit a final report for the 2007 phase II clinical trial to the FDA and HRPO and request a meeting with FDA to discuss the trial results.

3.1.3 Submit a protocol to the FDA and HRPO relating to the safety of a 30μg dose of LtSTA in naïve persons residing in the United States. The protocol also to include a provision to test a subset of ten individuals with four (4) 30μg doses to determine if negative to positive conversion occurs. Obtain informed consent from a local IRB and HRPO to conduct the trial.

3.1.4 Conduct the 30μg dose safety trial (item 3) and submit a final report to the FDA and HRPO.

3.1.5 Evaluate potential sites to conduct future studies in persons with *L. tropica* infection and test these individuals with a 30μg dose of LtSTA to compare their skin test response with the response observed in persons with *L. major* infection who were included in the phase II Sidi Bouzid trial.

3.1.6 Manufacture additional lots of LtSTA and test the stability of the product at a strength of 300μg per mL (30μg/0.1mL). Continue refinement of the manufacturing process under Good Manufacturing Practices.

3.2 Key Accomplishments

3.2.1 Analyzed data obtained from a phase II clinical trial conducted in 2007 in Sidi Bouzid, Tunisia.

The results of the phase II clinical trial were analyzed; the analysis revealed the following:

3.2.1.1 Twenty (20) adult volunteers with active cutaneous leishmaniasis (CL) were skin tested with four doses of LtSTA (10, 20, 40 80μg per 0.1mL). Each dose was administered to five subjects. A dose of 30μg was selected from a best-fit dose-response line based on these data.

3.2.1.2 Forty (40) adult volunteers with healed CL within the past 24 months were skin tested with the 30μg dose of LtSTA. Thirty-four (34) subjects reacted with a positive (≥5mm) induration response after 48 hours. Based on these data, LtSTA was shown to have an observed sensitivity of 85% in this population. The six (6) individuals with negative skin tests to the 30μg dose were retested with a 50μg dose. The 50μg dose elicited positive skin tests in all six (6) individuals. Lymphocyte profiles also were obtained for these individuals to check activity against *L. tropica* and *L. major*.

3.2.1.3 Forty (40) adult volunteers without known exposure to *L. major* were skin tested with a 30μg dose of LtSTA. Thirty-nine (39) subjects did not react to the product; one (1) subject had a positive skin test. Based on these findings, the observed specificity of LtSTA was 97%.

3.2.1.4 No serious local or systemic adverse events were observed in study participants. Local reactions to the skin test articles included itching, redness, swelling and minor pain at the skin test site.

3.2.2 Submission of a final report for the 2007 phase II clinical trial to the FDA and to HRPO.

The final report was submitted to the FDA and to HRPO on February 7, 2008. A copy of the report is included in the Appendix as Attachment 1.

A Type B meeting with the FDA was postponed until the results of a phase IIb trial are known. This trial is currently being conducted in San Diego, CA. Please see item 4 below.

3.2.3 Submission of a protocol to the FDA and HRPO relating to the safety and sensitizing properties of a 30μg dose of LtSTA in naïve persons resident in the United States.

The protocol that was submitted for review was modified to include 15, 30 and 50μg doses of LtSTA. The addition of a 15μg dose and a 50μg dose resulted from discussions with medical and scientific personnel at the U.S. Army. The protocol that was approved by Biomedical Research Institute of America (IRB) and HRPO for use in this study is included in the Appendix as Attachment 2.

3.2.4 Planned, organized and executed a phase IIb trial in which the safety and sensitizing properties of 15μg, 30μg and 50μg doses of LtSTA were evaluated.

Enrollment in this trial began in August 2008 following approval of study documents by the IRB and HRPO. At the present time, 51 subjects have signed the informed consent, 12 subjects failed to qualify, 9 subjects dropped, 25 subjects completed the study and 4 subjects are currently enrolled.

The protocol was amended to increase the number of subjects in the 30μg dose cohort to a maximum of 20 subjects to increase statistical power. The amended protocol (LtSTA-08 Rev03A) is included in the Appendix as Attachment 3.

A complete study report will be submitted to the U.S. Army after the phase IIb trial has been completed and the results have been properly analyzed. The results of the study to date are summarized as follows:

3.2.4.1 No serious adverse events have occurred in study subjects.

3.2.4.2 The 15μg dose of LtSTA was tested in twelve (12) subjects. The product did not induce sensitivity in nine (9) subjects who completed the trial. Three (3) subjects in this cohort were dropped from the trial prior to receiving the third injection of the antigen. Further evaluation of the 15μg dose will not be attempted unless the 30μg dose is found to be sensitizing.

3.2.4.3 The 50μg dose of LtSTA was tested in eleven (11) subjects. This dose induced sensitivity in two (2) subjects. One subject had a 11mm DTH response to the third intradermal injection and one subject had a 4mm DTH response to the third intradermal test. Sensitivity was not observed in nine subjects who completed the trial.

3.2.4.4 The 30μg dose was administered to twelve (12) subjects. Four (4) subjects dropped from the trial prior to receiving three doses of the antigen and were replaced with new volunteers. Eight (8) subjects have completed the trial and four subjects are awaiting testing of the third 30μg dose. Sensitization was not observed in the eight subjects that completed the trial.

3.2.4.5 Vital signs and laboratory results for study subjects have not raised concerns about the safety of LtSTA at concentrations of 15, 30 and 50μg when the product is administered intradermally.

3.2.5 Considered potential sites to conduct future studies in persons with L. tropica infection.

Discussions have been held with Dr. Max Grogl of the U.S. Army and Dr. Afif Ben Salah of the Pasteur Institut in Tunis, Tunisia regarding potential sites for a phase III clinical trial. No decision has been made as to the location and number of clinical sites that will be involved.

3.2.6 Refined manufacturing and quality testing for LtSTA following Good Manufacturing Practices.

Refinements in the manufacturing process continue to be made based on Allermed's production model. Developmental procedures are being converted to Standard Operating Procedures. Key steps in the manufacturing process are being validated. Special attention has been given to the (1) optimization of atmospheric growth conditions, (2) development and validation of a guinea pig potency assay, and (3) development and validation of a product identity test.

3.2.6.1 Optimization of Atmospheric Growth Conditions

The introduction of filtered air directly into the culture medium during the expansion of the *Leishmania tropica* parasite results in a significant increase in cell concentration. For example, the cell concentration of a typical Celstir culture is in the range of 5.0-6.0 x 10^7 promastigotes per mL. However, when pressurized air is introduced into the culture medium, the cell concentration increases to 1.5 x 10^8 promastigotes per mL, which is approximately a three-fold increase in the cell concentration.

3.2.6.2 LtSTA Guinea Pig Potency Assay

Allermed has developed a procedure that insures the production of manufactured lots of LtSTA that are comparable in potency to an internal reference standard (LRS). It is believed that a procedure based on the potency value of a production lot relative to the potency of a LRS will minimize the variability within the assays performed. Variation in this test can result from the dilution of the reagents, the guinea pigs used in the test, and/or the technicians performing the test, but because the effects are exerted on both the reference and production lots in parallel, the impact of these variables on the results of the test is minimized, if not completely eliminated. The relative potency test method and the validation protocol for this method are included in the appendix as Attachment 4.

3.2.6.3 LtSTA Identity Test

The identity test for LtSTA is an indirect ELISA based assay in which LtSTA specific rabbit antiserum is used to identify *Leishmania tropica* antigenic determinants. Goat anti-rabbit IgG secondary antibody. The reaction is read spectrophotometrically. A positive control (LRS) and two negative controls are run concurrently with the test lot of LtSTA. The identity test procedure and the validation protocol for the assay are included in the Appendix as Attachment 5.

3.3 Reportable Outcomes

3.3.1 LtSTA is safe to administer as a DTH antigen at intradermal doses up to 80μg. Serious adverse events have not been observed with LtSTA at these dose levels.

3.3.2 Dose-response testing of LtSTA results in a linear response to the antigen similar to the response observed with other DTH antigens.

3.3.3 A 30μg dose of LtSTA had an observed sensitivity of 0.85 in a population of forty *L.major* sensitized adult volunteers.

3.3.4 A 30μg dose of LtSTA had an observed specificity of 0.97 in a population of forty adult volunteers residing in a *L. major* endemic area.

3.3.5 A 50μg dose of LtSTA elicited a positive response in six *L. major* sensitized persons who were skin test negative to a 30μg dose of the antigen. The lymphocytes of these individuals demonstrated greater sensitivity to *L. major* antigen than to *L, tropica* antigen in a lymphocyte proliferation assay.

3.3.6 Induced sensitivity from repeat injections of a 50μg dose of LtSTA was observed in 18% (2/11) of Leishmania naïve adult volunteers.

3.3.7 The number and robustness of *L. tropica* promastigotes are increased by the introduction of air into the growth medium during culture.

3.3.8 A parallel line bioassay in *L. tropica* sensitized guinea pigs can be used as a potency assay for LtSTA.

3.3.9 An indirect ELISA assay using anti-*L. tropica* rabbit serum can be used as an identity test for LtSTA.

3.4 Conclusions

The studies that have been completed to date on LtSTA support the continued development of LtSTA at a concentration of 30μg/0.1mL. Product sensitivity is increased in a *Leishmania major* sensitized population if the dose is increased to 50μg/0.1mL. However, in a *Leishmania* naïve population, a 50μg dose induces sensitivity after two skin tests in some individuals. This finding is acceptable if LtSTA is intended to be used only once, but is unacceptable if the skin test antigen is intended to be used diagnostically in *Leishmania* naïve persons before and after entering a *Leishmania* endemic area. Sensitization after three skin tests has not been observed with a 30μg dose, but further investigation is needed to confirm this observation. A study is currently being conducted as an addendum to a phase IIb trial to evaluate the sensitizing properties of a 30μg dose in a larger number of *Leishmania* naïve subjects.

Refinements in the manufacture and quality testing of LtSTA are being made in accordance with current Good Manufacturing Practices. Developmetal procedures relating to increasing the yield of source material and the stability, identity and potency of the final product are being addressed and validated.

4. REFERENCES

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5. APPENDIX

5.1 Attachment 1 - Final Report of phase II clinical trial.

5.2 Attachment 2 - Protocol (LtSTA-08 Rev 3) for Phase IIb Clinical Trial.

5.3 Attachment 3 - Protocol Amendment (LtSTA-08 Rev 3A).

5.4 Attachment 4 - Relative Potency Test Method Validation Protocol.

5.5 Attachment 5 - Identity Test Method and Validation Protocol.

Attachment 1

Final Report of phase II clinical trial.

PHASE II STUDY FINAL REPORT

Dose-Response, Sensitivity and Specificity Testing of *Leishmania tropica* Skin Test Antigen for Cellular Hypersensitivity (LtSTA) in a *Leishmania major* Population

Protocol LtSTA-06, Revision 4

Sponsor
Allermed Laboratories, Inc.
7203 Convoy Court
San Diego, CA 92111

Research conducted under contract DAMD17-00-C-0030

United States Army Medical
Materiel Development Activity
Fort Detrick, MD 21702-5009

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1. ADMINISTRATIVE

1) **STUDY TITLE:**

Dose-Response, Sensitivity and Specificity Testing of *Leishmania tropica* Skin Test Antigen for Cellular Hypersensitivity (LtSTA) in a *Leishmania major* Population

2) **NAME OF INVESTIGATIONAL PRODUCT:**

Leishmania tropica Skin Test Antigen for Cellular Hypersensitivity (LtSTA)

3) **INDICATION STUDIED:**

Delayed-Type Skin Test Antigen to Assess Prior Exposure to *Leishmania major.*

4) **NAME OF SPONSOR:**

Allermed Laboratories, Inc.

5) **PROTOCOL NUMBER:**

LtSTA-06, Revision 4

6) **DEVELOPMENTAL PHASE OF STUDY:**

Phase II

7) **STUDY INITIATION DATE:**

February 13, 2007

8) **STUDY COMPLETION DATE:**

June 30, 2007

9) **NAME OF PRINCIPAL INVESTIGATOR:**

Afif Ben Salah, M.D., Ph.D.
Institut Pasteur de Tunis

10) **NAME OF SPONSOR SIGNATORY:**

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11) **CONDUCT OF STUDY UNDER GOOD CLINICAL PRACTICES (GCP)**

This study was conducted in compliance with current GCP including the archiving of essential documents.

12) **DATE OF REPORT:**
February 7, 2008

2. SYNOPSIS

Sponsor: Allermed Laboratories, Inc **Name of Finished Product:** *Leishmania tropica* Skin Test Antigen (LtSTA) **Active Ingredient:** *Leishmania tropica* Promastigote Antigen	BB IND 11822 Protocol LtSTA-06, Rev. 4 Feb 7, 2007 Tunisia	
Title of Study: Dose-Response, Sensitivity and Specificity Testing of *Leishmania tropica* Skin Test Antigen for Cellular Hypersensitivity (LtSTA) in a *Leishmania major* Population		
Investigator(s): Dr. Afif Ben Salah, M.D., Ph.D.		
Study Center: Institut Pasteur de Tunis		
Publication (reference): None		
Study Period: 6 months Date of first enrollment: 02/13/07 Date last completed: 06/30/07	Phase of Development: II	

Objectives:

1) Determine by skin test titration an appropriate dose of LtSTA to use as a skin test antigen with maximum sensitivity and specificity in a *Leishmania major* population.
2) Evaluate the dose identified in item 1 for sensitivity in adult volunteers with a history of cutaneous leishmaniasis (CL) within the past 24 months.
3) Evaluate the dose identified in item 1 for specificity in adult volunteers without a history of cutaneous leishmaniasis (CL).

Methodology:

1) Titration of LtSTA

   Four (4) doses of LtSTA (10, 20, 40, 80μg) were tested in adult subjects with active cutaneous leishmaniasis caused by *Leishmania major*. Each dose was administered intradermally to five subjects. These data were used to construct a dose-response line from which LtSTA concentrations eliciting a 15mm-18mm induration response was calculated. A concentration of 30μg was selected. This 30μg dose was tested for sensitivity and specificity as described below.

2) Sensitivity Testing

   Forty (40) adult volunteers with a history of CL caused by *L.major* within the past

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Name of Finished Product: *Leishmania tropica* Skin Test Antigen (LtSTA)		
Active Ingredient: *Leishmania tropica* Promastigote Antigen		

24 months were skin tested with 0.1mL of 30μg LtSTA. Skin tests were read 48 hours after the administration of the test article. Induration of $\geq$ 5mm was considered a positive DTH response.

3) Specificity Testing

Forty (40) adult volunteers without known exposure to *L.major* were skin tested with 0.1mL of 30μg LtSTA. Skin test procedures were the same as those described above under sensitivity testing

Number of Patients: Titration (20), Sensitivity (40), Specificity (40)

Diagnosis and Main Criteria for Inclusion:

1) Titration Group:
Volunteers with active cutaneous leishmaniasis (CL) were diagnosed from the appearance of cutaneous lesions, microscopic examination of lesion scrapings and isolation of the *Leishmania* parasite.
2) Sensitivity Group: Volunteers with a history of CL within the past 24 months were enrolled in the study based on the previous diagnosis of CL from clinical and laboratory observations recorded in medical records.
3) Specificity Group: Volunteers without a history of CL were enrolled in the study based on medical history and examination for evidence of leishmanial scars.

All volunteers were subjected to a physical examination and laboratory work-up. The criteria for enrollment varied between groups. See attached protocol for inclusion/exclusion criteria.

Test Product, Dose and Mode of Administration, Batch Number:
LtSTA is a clear, sterile solution containing the water extractables of *L.tropica* promastigotes. The product is standardized by protein content, stabilized with buffered saline and preserved with 0.4% phenol. The dose is 0.1mL administered intradermally in the forearm. The batch number (lot) of LtSTA used in the trial was XLtSTA014. The product was provided to the clinical site at a concentration of 120μg/0.1mL. It was diluted to the desired concentration by the principal investigator's staff.

Duration of Treatment:
Participants were skin tested on Visit 2 of the study. The results of skin tests were read after 48 hours ($\pm$ 4 hours) on Visit 3.

Reference Therapy, Dose and Mode of Administration, Batch Number:
See dose and administration above.

Sponsor: Allermed Laboratories, Inc	BB IND 11822 Protocol LtSTA-06, Rev. 4 Feb 7, 2007 Tunisia	
Name of Finished Product: *Leishmania tropica* Skin Test Antigen (LtSTA)		
Active Ingredient: *Leishmania tropica* Promastigote Antigen		

Criteria for Evaluation:

Efficacy: Efficacy of LtSTA as a diagnostic skin test antigen was based on the outcome of the DTH response. Induration less than 5mm at 48 hours demonstrated the absence of sensitivity or product specificity, depending upon the test group. Induration ≥ 5mm at 48 hours demonstrated the presence of sensitivity or lack of specificity, depending upon the test group.

Safety: The safety of LtSTA was based on the absence of local or systemic reactions associated with its use. Local reactions that were monitored included swelling, itching, pain, blistering and necrosis. Systemic responses included fever, chills, flu-like symptoms, hive, rash, and anaphylaxis.

Statistical Methods: Data obtained from the titration phase of the study were used to determine a dose-response line where x = log of LtSTA concentration and y = mm induration, with the best fit linear regression Y on X given by:

$Y = A + BX$, where B = slope and A = y-intercept

The skin test dose (D*) that elicits N mm induration obtained by:

D* = antilog of X, where $X = (N - A)/B$

Product sensitivity and specificity were evaluated with the Fisher's Exact Test using the one-sided 95% lower confidence limit with sensitivity equal to ≥ 0.80 and specificity equal to ≥ 0.85%.

SUMMARY – CONCLUSIONS

Efficacy Results

1) Twenty (20) adult volunteers with active cutaneous leishmaniasis (CL) were skin tested with four doses of LtSTA (10, 20, 40, 80μg per 0.1mL). Each dose was administered to five subjects. From a best-fit dose response line based on these data a dose of 30μg was selected (see Tables 1-4).

2) Forty (40) adult volunteers with healed CL within the past 24 months were skin tested with the 30μg dose of LtSTA. Thirty-four (34) subjects reacted with a positive (≥ 5mm) induration response after 48 hours. Based on these data, LtSTA was shown to have an observed sensitivity of 85% in this population (see Table 5). The six (6) individuals with negative skin tests to the 30μg dose were retested with a 50μg dose. The 50μg dose elicited positive skin tests in all six (6) individuals. Lymphocyte profiles also were obtained on these individuals to check if active against *L.tropica* or *L.major*.

Sponsor: Allermed Laboratories, Inc	BB IND 11822 Protocol LtSTA-06, Rev. 4 Feb 7, 2007 Tunisia	
Name of Finished Product: *Leishmania tropica* Skin Test Antigen (LtSTA)		
Active Ingredient: *Leishmania tropica* Promastigote Antigen		

3) Forty (40) adult volunteers without known exposure to *L.major* were skin tested with a 30μg dose of LtSTA. Thirty-nine (39) subjects did not react to the product; one (1) subject had a positive skin test. Based on these findings, the observed specificity of LtSTA was 97% (see Table 6).

Safety Results: No serious local or systemic adverse events were observed in study participants. Local reactions to the skin test articles were observed, including itching, redness, swelling and minor pain at the skin test site (see Table 7).

CONCLUSION:

At a dose of 30μg, the observed sensitivity of LtSTA in a *L.major* infected population was 85%. The observed specificity of LtSTA in non-infected subjects residing in a *L.major* endemic area was 97%. Subjects with previous CL who failed to react to a 30μg dose were skin test positive to a 50μg dose. Lymphocyte proliferation was greater in this group to extract of *L.major* than to LtSTA. This finding demonstrated a stronger cellular immune response to the homologous species than to *L.tropica.*

3. LIST OF ABBREVIATIONS AND DEFINITIONS OF TERMS:

AE	Adverse event – any untoward medical occurrence in a clinical study subject administered a pharmaceutical product and that does not necessarily have a causal relationship with this treatment.
CL	Cutaneous leishmaniasis
CPM	Counts per minute
CRF	Case Report Form
Diluent	Buffered Solution used to dilute LtSTA solution
DTH	Delayed-type hypersensitivity
Essential documents	Documents that individually and collectively permit evaluation of the conduct of a study and the quality of the data produced (see section 8 of ICH E6 Guideline for Good Clinical Practice).
GCP	Good Clinical Practices as outlined in ICH E6 Guidance Document
cGMP	Current Good Manufacturing Practice – 21 Code of Federal Regulations Part 211
HRPO	Human Research Protections Office
Informed Consent	A process by which a subject voluntarily confirms his/her willingness to participate in a clinical trial, after having been informed of all aspects of the trial. Informed consent is documented by means of a written, signed, and dated informed consent form.
IRB	Institutional Review Board
LMA	*Leishmania major* Antigen
LtSTA	*Leishmania tropica* Skin Test Antigen
Non-reacting dose	A dose that produces an area of edema < 5mm in diameter
PBMC	Peripheral blood mononuclear cells
Placebo	Buffered solution containing all the components of LtSTA except the parasite lysate.
PPD	Purified Protein derivative of *M. tuberculosis*
SAE	Serious adverse event
SI	Stimulation Index of lymphocytes
USAMRMC ORP HRPO	U. S. Army Medical Research and Materiel Command Office of Research Protections, Human Research Protections Office
USAMRMC HURO	U. S. Army Medical Research and Materiel Command Human Use Review Office

4. ETHICS

4.1 INSTITUTIONAL REVIEW BOARDS

Le Comité d ethique de l'Institute Pasteur de Tunis
13 Place Pasteur BP74
Belvedere 1002 Tunis, Tunisia

Human Research Protections Office (HRPO)
504 Scott Street
Fort Detrick, MD. 21703-5012

4.2 ETHICAL CONDUCT OF STUDY:

This study was conducted in accordance with ethical principles originating in the Declaration of Helsinki.

4.3 INFORMED CONSENT:

Consent to participate in this study was obtained by the principal investigator by interviewing each study volunteer in the presence of a second medical doctor and a quality assurance officer. During these interviews, the study procedures and risks were clearly explained to the volunteer and all questions posed by the volunteer were answered in detail. A copy of the informed consent document is included in the Appendix.

5. INVESTIGATORS AND STUDY ADMINISTRATION STRUCTURE:

Sponsor's Representative:		Dr. H.S. Nielsen, Jr., Ph.D.
Principal Investigator:		Dr. Afif Ben Salah, M.D., Ph.D.
Site Investigator:		Dr. Mongi Abdouli, M.D.
Medical Monitor:		Dr. Nissaf Ben Alaya Bouaafif, M.D.
Quality Assurance Officer:		Mr. Nahil Belhaj-Hamida
Parasitologist:		Mr. Amor Zaatour
Statistician:		Suresh Rastogi, Ph.D.
Study Monitors:		H.S. Nielsen, Jr. Ph.D. Mr. Masoud Ansari, M.S. Mrs. Cheryl Delaney

6. INTRODUCTION

Leishmaniasis is a common parasitic disease occurring throughout Africa, Asia, and Latin America (1-4). The life cycle of the *Leishmania* parasite is complex. The form of the parasite found in the insect vector, the promastigote, is normally transmitted to man by the bite of female sand flies. These promastigotes enter the cells of macrophage lineage by specific receptors. Once inside the mononuclear cell, the promastigotes round up into amastigotes, (the intracellular form of the parasite), divide in the parasitophorous vacuole, and infect other target cells. After a blood meal from an infected host, amastigotes are released into the gut of sand flies where they mature into infective promastigotes ready to repeat the cycle.

Leishmania infections do not always result in symptomatic or overt clinical disease (5-7). Immunity is largely cell-mediated. Tests designed to detect cell-mediated immune responses in exposed individuals are more likely to represent true measures of infection. The use of a skin test to detect delayed-type hypersensitivity (DTH) is a convenient, simple, and cost effective method to assess cell-mediated immune function in humans and can be used for large scale population surveys (8). Although there are other methods of detecting cell-mediated immunity, these methods are more difficult to perform and are not practical for screening large numbers of individuals.

For more than ten years, the U.S. Army has been involved in the development of diagnostics for leishmaniasis, including skin test antigens and molecular-based diagnostic products. Skin testing is commonly used in endemic countries to diagnose leishmaniasis because this testing is easy to perform and does not require complicated equipment. Several *Leishmania* skin test antigens developed by the U.S. Army have successfully undergone phase I clinical testing (9, 10).

Reports from Iraq have raised concerns regarding the potential impact of cutaneous leishmaniasis on military personnel. Over 800 U.S. troops deployed to the Middle East in support of Operation Iraqi Freedom developed cutaneous leishmaniasis. Additionally, blood donations from all military personnel that served in Iraq were deferred for a year because of the risk of transfusion-transmitted leishmaniasis.

Past experience with this parasite has highlighted several areas of concern that a tuberculin-like skin test antigen of *Leishmania* could address. The antigen is easy to administer and may be used diagnostically as a screening tool for undiagnosed cases in personnel returning from endemic areas. Improved methods also are needed to protect the health and blood supply of active and returning military personnel serving in *Leishmania* endemic regions of the world that are important to the national interests of the United States.

In 2000, the U.S. Army contracted with Allermed to develop an FDA approved skin test antigen of *L.tropica.* The product that is being developed by Allermed, *"Leishmania tropica* Skin Test Antigen for Cellular Hypersensitivity" (LtSTA) was evaluated for safety in 2005 in a phase I clinical trial. The present report presents the results of a phase II trial conducted in 2007 in Sidi Bouzid, Tunisia.

7. STUDY OBJECTIVES:

This study was undertaken to determine a suitable dose of LtSTA to use as a diagnostic test in identifying persons with past and present infection by *L.major**. Animal studies conducted by Allermed demonstrated that guinea pigs that had been sensitized to *L.major* were skin test positive to LtSTA. This observation suggested that the antigen might also elicit positive skin tests in humans infected with *L.major.* To evaluate this hypothesis, the following objectives were considered:

1) Determine a skin test dose of LtSTA that would elicit a 15mm DTH response in persons with active cutaneous leishmaniasis caused by *L.major.*

2) Evaluate the sensitivity of the skin test dose in persons with a history of *L.major* cutaneous leishmaniasis within the past 24 months.

3) Evaluate the specificity of the skin test dose in persons without a known history of cutaneous leishmaniasis.

* Although LtSTA is manufactured from *L.tropica*, the majority of cases of cutaneous Leishmaniasis that have occurred in U.S. Military personnel serving in Iraq and Afghanistan have been caused by *L.major.*

8. INVESTIGATIONAL PLAN

To achieve the objectives of the study it was necessary to evaluate LtSTA in a population of individuals residing in a geographic area in which leishmaniasis occurs. The use of DTH antigens prepared from one species of *Leishmania* as a means of detecting cellular hypersensitivity to other species of the parasite was reported by Agwale et al. (11). These investigators showed that 93% of Nigerian patients with cutaneous leishmaniasis (Old World) responded to a skin test antigen made from a mixture of New World species. In other populations, antigens prepared from *L.amazonensis* (a New World Species), elicited approximately the same percentage of positive skin tests as antigens made from *L.major* (Old World). These observations demonstrated the presence of cross-reactivity among *Leishmania* species and, specifically, the potential utility of using LtSTA as a skin test antigen in a heterologous population.

Animal studies conducted by Allermed demonstrated the presence of cross-reactivity between *L.major* and *L.tropica.* Guinea pigs sensitized to *L.major* were skin tested with LtSTA and found to have positive DTH reactions to the antigen. This finding was of particular interest in that the vast majority of *Leishmania* cases diagnosed in U.S. Military personnel serving in Afghanistan and Iraq were infected with *L.major.*

To evaluate LtSTA in *L.major* infected persons the study was conducted in Sidi Bouzid, Tunisia. The study site was established in 1992 in collaboration with WHO/TDR through a research strengthening grant to the Epidemiology Department of Institut de Tunis, Tunisia. The site is located in central Tunisia in the focus of cutaneous leishmaniasis, 250 kilometers away from Tunis. It is composed of a health structure directly linked to the Primary Health Care Direction of Sidi Bouzid. It consists of a parasitology laboratory, a space for logistics, communication with a fax machine and international phone line, study files and storage of drugs, and a room for patient's examination, treatment, and management. The study site has a full time staff consisting of two M.D. general practitioners, one nurse, and two drivers to transport patients to the site.

The clinical trial was designed to first determine a dose of LtSTA that could be used to identify persons with present or past infection with *L.major.* This was done by evaluating the skin test response to four concentrations of the antigen in persons with active CL. From the results of this research, a best fit dose-response line was constructed and the dose estimated to elicit a 15mm induration response was calculated. This dose (30μg) was evaluated for sensitivity and specificity in persons with and without a history of CL caused by *L.major.* A summary of the steps followed in conducting the trial is shown below:

1) Titration of LtSTA

Four (4) doses of LtSTA (10, 20, 40, 80μg) were tested in adult subjects with active cutaneous leishmaniasis caused by *Leishmania major*. Each dose was administered intradermally to five subjects. These data were used to construct a dose-response line from which an LtSTA concentration of 30μg was selected. This 30μg dose was tested for sensitivity and specificity as described below.

2) Sensitivity Testing

Forty (40) adult volunteers with a history of CL caused by *L.major* within the past 24 months were skin tested with 0.1mL of 30μg LtSTA. Skin tests were read 48 hours after the administration of the test article. Induration of $\geq$ 5mm was considered as a positive DTH response.

3) Specificity Testing

Forty (40) adult volunteers without known exposure to *L.major* were skin tested with 0.1mL of 30μg LtSTA. Skin test procedures were the same as those described above under sensitivity testing.

Based on the outcome of this trial, Allermed intended to continue the development of LtSTA at the concentration used in the trial, or adjust the dose higher or lower to provide maximum differentiation between *L.major* positive and *L.major* negative individuals.

8.1 POPULATION STUDIED

The Tunisian population is a mixture of Berbers (the original ethnic group), Arabs, Turks, and Europeans. This genetic and socio-cultural diversity is characteristic of the Tunisian population. The table below shows the relative importance of different ethnic groups in Tunisia:

Major Languages	Ethnic Groups
Arabic (official)	Arab-Berber > 98%
French	European < 1%
English	Other < 1%

The study population was located in the Governorate of Sidi Bouzid where cutaneous leishmaniasis is a serious public health problem. Sidi Bouzid is an endemic region for *L.major*. A total of 100 volunteers, ages 18-65 years, inclusive of either gender, were involved in the study. Volunteers were recruited from the local community by non-coercive means. Twenty volunteers with active cutaneous leishmaniasis (CL) were enrolled in the titration phase of the study. Forty volunteers with a history of CL within the past 24 months and forty volunteers with no history of active CL were enrolled in the sensitivity/specificity phase of the study.

8.2 SELECTION OF STUDY PARTICIPANTS

Leishmaniasis is a compulsory notifiable disease in Tunisia. Patient treatment is free at all primary health care centers. Patients with cutaneous leishmaniasis obtain care at the nearest Primary Health Care Centre (PHC) under the supervision of a general practitioner (MD). The recruitment process utilized this network of PHCs in the study area.

8.2.1 Diagnosis and Main Criteria for Inclusion:

1. Titration Group:

Volunteers with active cutaneous leishmaniasis (CL) were diagnosed from the appearance of cutaneous lesions, microscopic examination of lesion scrapings and isolation of the *Leishmania* parasite.

2. Sensitivity Group:

Volunteers with a history of CL within the past 24 months were enrolled in the study based on the previous diagnosis of CL from clinical and laboratory observations recorded in medical records.

3. Specificity Group:

Volunteers without a history of CL were enrolled in the study based on medical history and examination for evidence of leishmanial scars.

All volunteers were subjected to a physical examination and laboratory work-up. The criteria for enrollment are shown below in the inclusion/exclusion criteria.

8.2.2 Inclusion Criteria:

- Male or female in good health
- Age 18-65
- Confirmed history of leishmaniasis in the active and healed leishmaniasis group.
- No history of leishmaniasis in the control group.
- No previous skin test with *Leishmania* antigen

8.2.3 Exclusion Criteria

- History of adult atopic dermatitis, contact dermatitis to multiple agents, unexplained urticaria, or asthma
- Active allergic rhinitis or conjunctivitis
- Allergy to phenol, pharmaceutical detergents, Tween 80® or glycerol
- Medications: steroids, antihistamines, cimetidine, immunosuppressants within 3 months
- Splenectomy
- Active medical disease*
- Pregnancy or lactating
- Active cutaneous leishmaniasis with scar(s) i.e. possible re-infections and recidivist leishmaniasis cases
- Immunization within 4 weeks
- Anergy on DTH testing (less than 5mm of induration)
- Abnormal screening lab results

* **Active Medical Disease:** Any active physical or psychiatric condition that may increase the risks associated with participation in the study or interferes with the interpretation of study results. Included chronic medical illnesses are: cardiovascular disease, renal insufficiency, chronic respiratory illness, cirrhosis, chronic hepatitis, chronic pancreatitis, chronic diarrhea, malnutrition, malignancy, autoimmune disease, and asthma.

8.2.4 Non-Qualifying Volunteers

Persons who signed the informed consent, but who did not qualify for enrollment in the study due to: (1) their failure to meet all inclusion/exclusion criteria, or (2) other documented conditions considered sufficient by the principal investigator or medical monitor to warrant the exclusion of the volunteer, were informed that they could not participate in the study. The reason for exclusion was explained to the volunteer and appropriate recommendations were provided by the principal investigator in consultation with the medical monitor. All records, laboratory reports, etc., relating to the enrollment process for the volunteer were kept and maintained with other study documents.

8.2.5 Volunteers with Concomitant or Intercurrent Illness Detected during Screening

Persons with concomitant or intercurrent illness that was detected during enrollment screening were excluded from participation in the study. These individuals were considered non-qualifying volunteers. The procedure that was followed was the same as those described above for non-qualifying volunteers.

8.2.6 Withdrawal Criteria

Volunteers were allowed to withdraw from the study at any time without prejudice or loss of benefits to which they were entitled. The study was designed to remove participants from the study by the principal investigator or the medical monitor if their continued participation was believed to be injurious to their health and well being.

8.2.7 Method of Assigning Volunteers to Study Groups

Volunteers with active CL were recruited from the network of Primary Health Care Centers (PHC). They were enrolled in the titration phase of the study as they presented themselves at the study site.

Volunteers with a history of CL within the past 24 months were identified from the PHC case records and enrolled in the study as they presented themselves at the study site.

Volunteers without a history of CL were obtained from the community and enrolled in the study as they presented themselves at the study site.

The age range, ethnicity and gender of volunteers enrolled in the trial were as follows:

	Age Range	Ethnicity	Gender		N
			M	F	
Active CL [1]	21-63	Arabic	9	11	20
Healed CL [2]	21-63	Arabic	14	26	40
No CL [3]	21-59	Arabic	13	27	40

(1) Dose-Response Group

(2) Sensitivity Group

(3) Specificity Group

8.2.8 Subjects contacted during the course of the study (i.e. number recruited, enrolled, withdrawn by principal investigator, discontinued by subject)

During the first phase of the study (dose-response phase), 37 volunteers were screened of which 20 were enrolled and received one of four increasing concentrations of LtSTA (10μg, 20μg, 30μg, and 80μg). The 17 subjects that were not enrolled were excluded for the following reasons: 4 refused to consent, 3 were under the limit of age, 2 had a history of allergy, 1 showed abnormal CBC, 1 was a breastfeeding woman, 1 refused to continue the consent, 1 showed a non-active lesion and 4 revealed other exclusion criteria (e.g. urticaria, asthma, skin allergy and food allergy).

During the second phase of the study (sensitivity and specificity), 129 volunteers were screened, 49 were excluded and 80 were enrolled. The reasons of exclusion were as follows: 9 refused the test and withdrew their consent, 9 had a history of leishmaniasis among those that were thought to be unexposed, 6 showed abnormal laboratory tests, 5 had a past history of allergy, 3 refused to consent, 3 refused the blood sampling for laboratory tests, 2 were unable to consent and were withdrawn by the principal investigator, 12 had other reasons of exclusion (1 breast feeding, 1 pregnancy, 3 eczema, 4 urticaria, 3 asthma).

One hundred (100) volunteers were enrolled in the study: 20 volunteers were included in the dose-response study and 80 volunteers were included in the sensitivity and specificity phase of the study.

8.2.9 Deviations

One deviation from the protocol was noticed and reported on March 21st, 2007 to the Chairman of the Medical Ethical Committee, Institut Pasteur de Tunis, 13 Place Pasteur BP, 74 Belvedere 1002, Tunis, Tunisia and the Human Research Protections Office (HRPO) 504 Scott Street, Fort Detrick, MD. 21702-5012, phone (301)-619-2165.

Twenty subjects were tested in the dose-response part of the protocol with each dose (10, 20, 40, 80μg) being evaluated in five subjects. All five individuals had positive reactions to the dose tested, except one person in cohort 4 (80μg). This subject did not react to the antigen and, upon further investigation, was found to have recidivist leishmaniasis which is known to alter the DTH response to *Leishmania* antigen. Since recidivist disease is listed in the exclusion criteria, this inclusion was a deviation to the protocol. As a corrective action, the principal investigator reviewed carefully with the study team all the inclusion/exclusion criteria to prevent future deviations.

8.3 INVESTIGATIONAL PRODUCTS

The test articles LtSTA and controls were delivered to the clinical site in temperature monitored containers. All test reagents were stored at 2–8°C at the study site. A description of the investigational products follows:

8.3.1 Description of Product

LtSTA

LtSTA is a clear solution formulated to the desired protein concentration with 0.85% sodium chloride, 0.4% phenol, 0.01% Tween-80®, and 1% glycerin in a 25mM phosphate buffer. For this study, LtSTA was supplied at a target concentration of 120μg/0.1mL protein (acceptable limits 110μg/0.1mL to 130μg/0.1mL) in a 10 dose vial. Dilutions of LtSTA were made per the "Procedure for the Preparation of LtSTA Dilutions" in the study protocol.

Placebo

The placebo control was manufactured by Allermed according to approved procedures and under cGMP. The *Leishmania tropica* Skin Test Placebo was formulated to contain all the components of LtSTA except the parasite lysate. The placebo consisted of 0.85% sodium chloride, 0.4% phenol, 0.01% Tween-80®, and 1% glycerin in a 25mM phosphate buffer.

Saline Control

A saline control containing 0.9% sodium chloride was purchased as a commercial product. This material was stored per the manufacturer's specifications at the clinical site.

LtSTA Diluent

The LtSTA Diluent was manufactured by Allermed according to approved procedures and under cGMP. Diluent contained 0.85% sodium chloride, 0.4% phenol, 0.01% Tween-80®, and 1% glycerin in a 25mM phosphate buffer.

Anergy Panel (DTH Controls)

Candin® and Trichophyton allergenic extract (1:500 w/v) were used as DTH controls. Candin® is an FDA approved product manufactured by Allermed Laboratories, Inc. that can be used to detect delayed-type hypersensitivity to the yeast *Candida albicans*.

Trichophyton is an FDA approved allergenic extract with DTH properties, manufactured by Allermed Laboratories, Inc. This extract is manufactured from the fungi *Trichophyton rubrum* and *Trichophyton mentagrophytes*, causative agents of dermatophytosis of the hair, nails, and skin. It was expected that 70 to 80% of trial volunteers with functional cellular immunity would produce a positive DTH response to one or both of the positive control antigens.

8.3.2 **Investigational Product Accountability**

All study products and drugs for anaphylaxis were logged into the clinic on arrival from Allermed and kept in a secure location at the clinical site. A Chain of Custody Form accompanied the investigational products and became part of the study file. A written log was maintained and only investigators removed the products from storage for use in the study. The test articles were stored at 2 - 8°C; those not used within the study were discarded as medical waste, as appropriate. The lot numbers of all discarded vials were recorded in the log maintained at the clinic.

8.3.3 **Blinding of LtSTA and Controls**

LtSTA, placebo, saline and the two DTH control antigens (Candin® and Trichophyton) were removed from sterile vials using 1.0mL tuberculin syringes. The dose was 0.1mL for each reagent. Each filled syringe was labeled with the letters RP, RM, RD, LP, LM, or LD to signify the placement location of the reagent, i.e. a syringe labeled RD was administered on the distal portion (near the wrist) of the right forearm. This procedure was performed by a technician and over watched by a second individual to ensure accuracy. The filled syringes were then transported to a second room where the principal investigator administered the products intradermally according to the designated location shown on the syringe label. At no time did the principal investigator performing the skin test have any knowledge of the identity of the contents in the labeled syringes. The sequence of numbering was done following a randomized procedure.

9 STUDY RESULTS

9.1 DOSE-RESPONSE TITRATION OF LtSTA

The phase I safety trial conducted by Allermed in 2005 involved 32 healthy adult volunteers without known previous exposure to *Leishmania* parasites. Four doses of LtSTA (20, 40, 80, and 120μg) were evaluated for safety in terms of local and systemic adverse events associated with the intradermal injection of 0.1mL of the antigen. No serious adverse events were observed in the phase I trial. However, in two of eight volunteers the 120μg dose caused an induration response after two weeks which resembled a positive DTH reaction. This observation was believed to be the result of retained antigen at the skin test site to which the individuals developed sensitivity. Based on these findings, Allermed elected to use doses of 10, 20, 40 and 80μg in the dose-response titration of LtSTA in persons with active CL. The results of this investigation are summarized in Tables 1-4.

Table 1: Delayed – Type Hypersensitivity Skin Test Response to Four Doses (10, 20, 40, and 80μg) of *Leishmania tropica* Skin Test Antigen (LtSTA) in Adult Volunteers with Active Cutaneous Leishmaniasis caused by *Leishmania major*

Subject Initials	Volunteer Study I.D. No.	Age	Sex	Active Cutaneous Leishmaniasis		Dose		48 Hour Skin Test Reaction (Induration mm)		
				Yes	No	Group	μg	LtSTA	Saline	Placebo
MIMA	L001	21	F	√		1	10	6.5	Ø	Ø
JABE	L003	49	M	√		1	10	10	Ø	Ø
JAWR	L004	63	F	√		1	10	14	Ø	Ø
JAIB	L005	22	F	√		1	10	16	Ø	Ø
ZAHA	L006	34	F	√		1	10	8.5	Ø	Ø
MASA	L002	30	F	√		2	20	16	Ø	Ø
JAHA	L007	47	M	√		2	20	20	Ø	Ø
FAAB	L008	42	M	√		2	20	9.5	Ø	Ø
LEFA	L009	31	F	√		2	20	10	Ø	Ø
KARI	L010	27	M	√		2	20	16	Ø	Ø
HAAM	L011	32	M	√		3	40	17.5	Ø	Ø
GAZI	L012	42	F	√		3	40	17.5	Ø	Ø
TIKH	L014	28	M	√		3	40	25	Ø	Ø
HAHA	L015	30	M	√		3	40	21	Ø	Ø
BOMO	L016	22	M	√		3	40	15	Ø	Ø
DHAL	L017	61	M	√		4	80	21.5	Ø	Ø
GAMO	L018	28	F	√		4	80	25.5	Ø	Ø
SLJI	L019	21	F	√		4	80	21	Ø	Ø
RBAB	L020	39	M	√		4	80	Ø	Ø	Ø
KHMA	L021	35	F	√		4	80	32	Ø	Ø

Legend:
√ = a previous diagnosis of Leishmaniasis is indicated either negative or positive.
Ø = negative result
mm = sum of induration measuring two diameters ÷ 2

Tables 2–4 summarize the 48 hour induration response for each subject, the mean diameter of the response by dose and the estimated induration by dose calculated from the dose-response data.

Table 2: LtSTA Doses and Individual Induration Responses

Dose (mg/0.1mL)	Volunteer	Induration (mm)	Dose (mg/0.1mL)	Volunteer	Induration (mm)
10	1	6.5	40	11	17.5
	2	10.0		12	17.5
	3	14.0		13	25.0
	4	16.0		14	21.0
	5	8.5		15	15.0
20	6	20.0	80	16	25.5
	7	16.0		17	21.5
	8	9.5		18	21.0
	9	10.0		19	32.0
	10	16.0			

Table 3: Mean Induration by Dose

Dose (µg/0.1mL)	Log (Dose)	Mean Induration (mm)
10	1.0	10.4
20	1.3	14.3
40	1.6	19.2
80	1.9	25.0

Doses were converted to logarithm to the base 10. The best fit linear regression Y on X was calculated, where X is log (dose) and Y is the average induration at that dose. The results were:

Slope B = 16.2 Y-intercept A = - 6.3 and R-squared = 0.99

The slope was highly significantly different from zero with p-value = 0.004. R-squared was 0.99. Using this linear regression line, Y = - 6.3 + 16.2 X, the log (dose) corresponding to various induration sizes from 15mm to 18mm was calculated. These data are shown in Table 4 below:

Table 4: Estimated Doses

Induration (mm)	Log (Dose)	Dose (µg/0.1mL)
15	1.314	21
16	1.376	24
17	1.438	27
18	1.499	32

Induration was not observed to either the saline control or to placebo in the twenty (20) subjects with active CL who participated in the titration of LtSTA.

Although the targeted induration response was 15mm with a corresponding dose of 21μg/0.1mL, a dose of 30μg/0.1mL (approximately 18mm) was selected for further study. Part of the reasoning behind this decision was based on the observation that the 20μg/0.1mL dose (Table 2) elicited induration responses in two subjects of 9.5mm and 10mm which were deemed to be borderline small. The 30μg dose was evaluated for sensitivity and specificity in persons with active CL within the past 24 months and in persons without a history of CL.

9.2 SENSITIVITY OF THE 30μg DOSE OF LtSTA

The sensitivity of LtSTA (detect prior infection with *L.major*) was evaluated in forty (40) volunteers with a history or cured CL caused by *L major* within the past 24 months. This cohort consisted of fourteen (14) male and twenty-six (26) female adults between the ages of 21 and 63. Each volunteer received five intradermal injections consisting of LtSTA, saline, placebo, Candin®, and Trichophyton. The test articles were administered on a blinded basis and the reading of test results was blinded. The results of this work are summarized below and presented in detail in Table 5.

LtSTA	Saline	Placebo	Candin®	Trichophyton
34/40*	0/40	0/40	39/40	18/40

*numerator = number of persons with induration $\geq$ 5mm at 48 hours
denominator = number of persons tested

Positive DTH skin tests to 30μg LtSTA were observed in 34/40 (85%) of the cohort. The two positive control antigens, Candin® and Trichophyton, reacted in 39/40 (97%) and 18/40 (45%), respectively, and saline and placebo did not elicit a positive induration response. The six (6) subjects who did not react to 30μg LtSTA were further studied as described in sections 9.4 and 9.5.

Table 5: Delayed – Type Hypersensitivity Skin Test Response to 30µg *Leishmania tropica* Skin Test Antigen in Adult Volunteers with a History of Cutaneous *Leishmania* Caused by *Leishmania major*

Subject #	Subject Initials	Volunteer I.D. No.	Age	Race	Sex	CL within past 24 Months		48 Hour Skin Test Reaction (Induration mm)				
						Yes	No	LtSTA	Saline	Placebo	Candin®	Trichophyton Extract
1	MINE	SSL001	39	Ar	M	√		**10.5**	Ø	Ø	11	17
2	TIMO	SSL002	48	Ar	M	√		**13.5**	Ø	Ø	12.5	5.5
3	SOZA	SSL003	42	Ar	M	√		**12.5**	Ø	Ø	12.5	13
4	FAMA	SSL004	25	Ar	F	√		**12**	Ø	Ø	10.5	20
5	TISA	SSL005	22	Ar	F	√		**24.5**	Ø	Ø	15	Ø
6	MIJA	SSL006	29	Ar	F	√		**11**	Ø	Ø	10	10
7	MIBA	SSL007	26	Ar	M	√		**19**	Ø	Ø	13	9.5
8	MIZO	SSL008	42	Ar	F	√		**17**	Ø	Ø	8.5	Ø
9	JAMO	SSL009	41	Ar	F	√		**17**	Ø	Ø	11.5	Ø
10	TIZO	SSL010	49	Ar	F	√		**18**	Ø	Ø	12	8.5
11	FAMO	SSL011	54	Ar	M	√		**23.5**	Ø	Ø	11	Ø
12	MIMOA	SSL014	33	Ar	M	√		**18**	Ø	Ø	9	12
13	MIMO	SSL015	37	Ar	M	√		**22**	Ø	Ø	18.5	7.5
14	KAMO	SSL016	21	Ar	F	√		Ø	Ø	Ø	13.5	Ø
15	MRBA	SSL017	21	Ar	F	√		**13**	Ø	Ø	9.5	Ø
16	JAHO	SSL018	22	Ar	F	√		**17**	Ø	Ø	10	12.5
17	KHRG	SSL019	59	Ar	F	√		Ø	Ø	Ø	15	Ø
18	HAHAn	SSL020	55	Ar	F	√		**15**	Ø	Ø	13	Ø
19	DHNA	SSL021	28	Ar	M	√		**21**	Ø	Ø	17	Ø
20	KHZI	SSL022	55	Ar	F	√		**9.5**	Ø	Ø	15	Ø
21	CHSI	SSL023	25	Ar	F	√		**16.5**	Ø	Ø	10	Ø
22	HAMO	SSL025	44	Ar	F	√		**11**	Ø	Ø	13.5	Ø
23	ABBR	SSL026	48	Ar	M	√		Ø	Ø	Ø	14.5	12
24	KHMO	SSL027	51	Ar	M	√		**18.5**	Ø	Ø	26	15.5
25	KHAM	SSL028	41	Ar	M	√		**15.5**	Ø	Ø	29.5	17.5
26	HAEM	SSL029	52	Ar	F	√		**14.5**	Ø	Ø	16	Ø
27	HAMAb	SSL030	56	Ar	F	√		**10**	Ø	Ø	14	Ø
28	HAHAm	SSL031	21	Ar	F	√		**6**	Ø	Ø	16	Ø

Legend:
√ = a previous diagnosis of Leishmaniasis is indicated either negative or positive.
Ø = negative result
mm = sum of induration measuring two diameters ÷ 2

Abbreviations:
Ar = Arabic

Table 5 (continued): Delayed – Type Hypersensitivity Skin Test Response to 30µg *Leishmania tropica* Skin Test Antigen in Adult Volunteers with a History of Cutaneous *Leishmania* Caused by *Leishmania major*

SUBJECT #	SUBJECT INITIALS	VOLUNTEER I.D. NO.	AGE	RACE	SEX	CL WITHIN PAST 24 MONTHS		48 HOUR SKIN TEST REACTION (INDURATION mm)				
						YES	NO	LtSTA	SALINE	PLACEBO	CANDIN®	TRICHOPHYTON EXTRACT
29	GAAM	SSL032	53	Ar	M	√		**13**	Ø	Ø	7	20
30	MADA	SSL034	37	Ar	F	√		**23**	Ø	Ø	14.5	17.5
31	MAHS	SSL035	57	Ar	M	√		Ø	Ø	Ø	16	Ø
32	ABEL	SSL036	51	Ar	F	√		**10**	Ø	Ø	18	Ø
33	DONA	SSL038	42	Ar	F	√		Ø	Ø	Ø	19.5	10.5
34	RIZO	SSL039	41	Ar	F	√		Ø	Ø	Ø	14	Ø
35	MIOL	SSL040	31	Ar	F	√		**18**	Ø	Ø	19	9
36	DHZI	SSL041	58	Ar	F	√		**17**	Ø	Ø	13.5	16.5
37	BEGA	SSL042	25	Ar	F	√		**7**	Ø	Ø	Ø	Ø
38	BEZA	SSL043	32	Ar	F	√		**16**	Ø	Ø	15	12
39	BEHA	SSL044	50	Ar	F	√		**21.5**	Ø	Ø	13	Ø
40	BEMO	SSL045	63	Ar	M	√		**15**	Ø	Ø	15.5	Ø

LEGEND:
√ = a previous diagnosis of Leishmaniasis is indicated either negative or positive.
Ø = negative result
mm = sum of induration measuring two diameters ÷ 2

ABBREVIATIONS:
Ar = Arabic

9.3 SPECIFICITY OF THE 30μg DOSE OF LtSTA

The specificity (failure of LtSTA to elicit a positive DTH skin test response in persons without previous exposure to *L.major*) was evaluated in forty adults who lived in a *L.major* endemic area, but who had no known exposure to *Leishmania major*. This cohort consisted of thirteen (13) male and twenty-seven (27) female Arabic adults between the ages of 21 and 59. Each volunteer received five intradermal injections consisting of LtSTA, saline, placebo, Candin®, and Trichophyton. The test articles were administered on a blinded basis and the reading of test results was blinded. The results of this work are summarized below and presented in detail in Table 6.

LtSTA	Saline	Placebo	Candin®	Trichophyton
1/40*	0/40	0/40	40/40	8/40

* numerator = number of persons with induration ≥ 5mm at 48 hours
denominator = number of persons tested

Thirty-nine (39) subjects (97%) without a history of past *L.major* infection did not react to the 30μg dose of LtSTA. One (1) subject BOMO had an induration response of 8.5mm after 48 hours which was interpreted as a positive DTH skin test. Saline and placebo did not elicit positive skin tests, whereas Candin® and Trichophyton reacted in 100% and 20%, respectively, of the individuals tested. Subject BOMO was further questioned regarding potential exposure to *L.major* and it was learned by the principal investigator that this person had spent several months during the season in which *L.major* is transmitted to humans in a region of Tunisia which is highly endemic for *L.major*. Further investigation of this subject's peripheral blood revealed a strong lymphoproliferative response to *L.major* (see section 9.5).

Table 6: Delayed – Type Hypersensitivity Skin Test Response to 30µg *Leishmania tropica* Skin Test Antigen in Adult Volunteers Without Known Exposure to *Leishmania major*

Subject #	Subject Initials	Volunteer I.D. No.	Age	Race	Sex	CL within past 24 Months		48 Hour Skin Test Reaction (Induration mm)				
						Yes	No	LtSTA	Saline	Placebo	Candin®	Tricho. Ex.
41	NCFA	SSL046	47	Ar	F		√	Ø	Ø	Ø	14.5	Ø
42	BOSM	SSL047	43	Ar	F		√	Ø	Ø	Ø	18.5	Ø
43	BOYO	SSL048	31	Ar	M		√	Ø	Ø	Ø	17.5	Ø
44	AKSO	SSL049	50	Ar	F		√	Ø	Ø	Ø	11	Ø
45	NCMO	SSL050	26	Ar	M		√	Ø	Ø	Ø	14	Ø
46	YONI	SSL052	25	Ar	F		√	Ø	Ø	Ø	10	Ø
47	JESO	SSL053	27	Ar	F		√	Ø	Ø	Ø	13	Ø
48	GHFA	SSL054	27	Ar	M		√	Ø	Ø	Ø	17	Ø
49	JAOL	SSL055	21	Ar	F		√	Ø	Ø	Ø	9.5	Ø
50	SANO	SSL056	21	Ar	F		√	Ø	Ø	Ø	13	Ø
51	ZAAB	SSL057	50	Ar	M		√	Ø	Ø	Ø	13	Ø
52	BOZA	SSL058	27	Ar	F		√	Ø	Ø	Ø	15.5	Ø
53	BOAM	SSL059	55	Ar	F		√	Ø	Ø	Ø	8	8
54	BALE	SSL060	25	Ar	F		√	Ø	Ø	Ø	12.5	Ø
55	MEIN	SSL061	27	Ar	F		√	Ø	Ø	Ø	9	Ø
56	MAHE	SSL062	28	Ar	M		√	Ø	Ø	Ø	13.5	Ø
57	NEBO	SSL064	26	Ar	M		√	Ø	Ø	Ø	13	Ø
58	RANA	SSL066	23	Ar	F		√	Ø	Ø	Ø	12	Ø
59	MBHO	SSL067	24	Ar	F		√	Ø	Ø	Ø	12.5	13
60	SASA	SSL068	27	Ar	F		√	Ø	Ø	Ø	16	Ø
61	BAME	SSL069	25	Ar	F		√	Ø	Ø	Ø	11	7
62	BERA	SSL072	22	Ar	F		√	Ø	Ø	Ø	11	Ø
63	YOMO	SSL073	23	Ar	F		√	Ø	Ø	Ø	13.5	Ø
64	MIHA	SSL074	22	Ar	F		√	Ø	Ø	Ø	11.5	Ø
65	CHNA	SSL075	28	Ar	M		√	Ø	Ø	Ø	15.5	Ø
66	OUWR	SSL076	28	Ar	F		√	Ø	Ø	Ø	15	Ø
67	BOJA	SSL077	39	Ar	F		√	Ø	Ø	Ø	17	18.5
68	BOMA	SSL078	55	Ar	F		√	Ø	Ø	Ø	14.5	Ø

Legend:
- √ = a previous diagnosis of Leishmaniasis is indicated either negative or positive.
- Ø = negative result
- mm = sum of induration measuring two diameters ÷ 2

Abbreviations:
- LtSTA = *Leishmania tropica* Skin Test Antigen
- Tricho Ex. = Trichophyton Extract
- CL = Cutaneous Leishmaniasis
- Ar = Arabic

Table 6 (continued): Delayed – Type Hypersensitivity Skin Test Response to 30µg *Leishmania tropica* Skin Test Antigen in Adult Volunteers Without Known Exposure to *Leishmania major*

Subject #	Subject Initials	Volunteer I.D. No.	Age	Race	Sex	CL within past 24 Months		48 Hour Skin Test Reaction (Induration mm)				
						Yes	No	LtSTA	Saline	Placebo	Candin®	Tricho. Ex.
69	BODA	SSL079	38	Ar	F		√	Ø	Ø	Ø	16.5	Ø
70	BOSAf	SSL080	36	Ar	F		√	Ø	Ø	Ø	13	Ø
71	BAMO	SSL081	44	Ar	M		√	Ø	Ø	Ø	13.5	Ø
72	NCHO	SSL082	30	Ar	M		√	Ø	Ø	Ø	14	Ø
73	BODH	SSL083	59	Ar	F		√	Ø	Ø	Ø	12	12.5
74	BOMO	SSL084	42	Ar	M		√	8.5	Ø	Ø	13	Ø
75	NCMH	SSL085	51	Ar	M		√	Ø	Ø	Ø	13.5	9
76	NCAI	SSL086	31	Ar	F		√	Ø	Ø	Ø	15	Ø
77	BOAL	SSL087	46	Ar	M		√	Ø	Ø	Ø	14.5	17
78	NCBE	SSL088	32	Ar	M		√	Ø	Ø	Ø	11	17.5
79	BOSE	SSL089	47	Ar	F		√	Ø	Ø	Ø	17	Ø
80	BOTOu	SSL090	39	Ar	F		√	Ø	Ø	Ø	11	Ø

Legend:
- √ = a previous diagnosis of Leishmaniasis is indicated either negative or positive.
- Ø = negative result
- mm = sum of induration measuring two diameters ÷ 2

Abbreviations:
- LtSTA = *Leishmania tropica* Skin Test Antigen
- Tricho Ex. = Trichophyton Extract
- CL = Cutaneous Leishmaniasis
- Ar = Arabic

9.4 SKIN TESTS WITH 50μg LtSTA

Under an amendment to the protocol, six subjects that had histories of CL within the past 24 months, but who were skin test negative to 30μg LtSTA were retested with LtSTA at a dose of 50μg. The results of this investigation are shown below in Table 7.

Table 7: Results of Skin Tests with 30μg and 50μg LtSTA

Study Number	Subject Initials	LtSTA 30μg	LtSTA 50μg	Mean
SSL091	MAHS	0 x 0	10 x 16	13.0
SSL092	DONA	0 x 0	18 x 21	19.5
SSL093	RIZO	0 x 0	14 x 15	14.5
SSL094	KHRG	0 x 0	20 x 23	21.5
SSL095	KAMO	0 x 0	15 x 19	17.0
SSL096	ABBR	0 x 0	9 x 11	8.0

Positive skin tests to a 50μg dose of LtSTA ranged from 8.0mm to 21.5mm compared to negative induration response to the 30μg dose of the antigen. These data were compared to the lymphocyte response of these individuals as described in section 9.5 below.

9.5 LYMPHOCYTE PROLIFERATION EXPERIMENTS

The proliferation of lymphocytes obtained from peripheral blood samples were compared between CL positive subjects with negative skin tests to 30μg LtSTA and a control group of subjects who were CL positive and had positive skin tests to 30μg LtSTA. Each group consisted of six subjects: Group 2 subjects were matched for age and gender against Group 1 subjects. The methods used in lymphoproliferation experiments were the same as those described by Sassi et al. (12).

Group 1: CL positive with negative skin tests to 30μg LtSTA (MAHS, ABBR, DONA, RIZO, KHRG, and KAMO).

Group 2: CL positive with positive skin tests to 30μg LtSTA (TIMO, JAMO, MIZO, FAMO, TIZO, and TISA).

Because *L.major* was the causal agent of the CL observed in all of these individuals an antigen preparation of *L.major* (LMA) and PPD were used as antigen controls for LtSTA in these experiments. The results of this work revealed the following:

1) Two subjects in Group 1 (MAHS, ABBR) had poor lymphocyte responses to both *Leishmania* antigens, but were strongly positive to PPD.

2) Four subjects in Group 1 (DONA, RIZO, KHRG and KAMO) had lymphocyte

responses to both antigens, but the response to LtSTA was less than 50% as strong as the response to *L.major* antigen (LMA). All four subjects responded strongly to PPD.

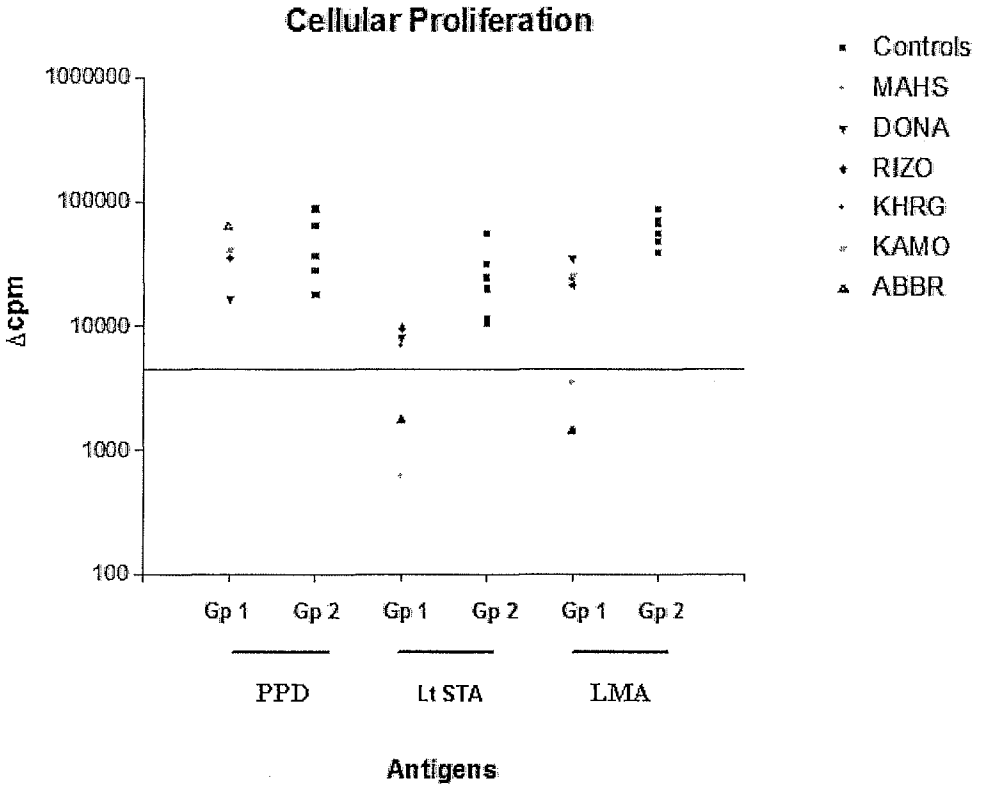
3) The six subjects in Group 2 had lymphocyte responses to both antigens, but the response to LtSTA was less than 50% as strong as the response to *L.major* antigen (LMA). All six subjects responded strongly to PPD.

Subjects in Group 1 also were re-skin tested with a 50μg dose of LtSTA prior to obtaining blood samples for the lymphocyte proliferation assays. Skin testing occurred approximately three months after the initial skin test with the 30μg dose. All six subjects had positive DTH skin tests to the 50μg dose. These results and the lymphocyte proliferation results are summarized in Table 8. Figure 1 shows the results of lymphocyte proliferation to PPD, LtSTA and *L.major* (LMA).

Table 8: Results of Skin Tests with 30μg and 50μg LtSTA and Lymphoproliferative Responses to *L.major* and *L.tropica* Antigens of Group 1 Subjects; Skin Test Results with 30μg LtSTA and Lymphoproliferative Responses of Group 2 Subjects also Shown.

Study Number	Subject Initials	LtSTA 30μg	LtSTA 50μg	(*L.tropica* Ag.) δ CPM/Stimulation Index	(*L.major* Ag.) δ CPM/Stimulation Index
Group 1 (Subjects with a negative skin test to 30μg LtSTA)					
SSL091	MAHS	0 x 0	16 x 10	6/1	3,434/2
SSL092	DONA	0 x 0	21 x 18	7,866/7	38,262/32
SSL093	RIZO	0 x 0	15 x 14	9,581/6	21,366/15
SSL094	KHRG	0 x 0	23 x 20	6,767/14	21,187/45
SSL095	KAMO	0 x 0	19 x 15	8, 062/6	21,473/16
SSL096	ABBR	0 x 0	11 x 9	1,161/1	815/1
Group 2 (Subjects with a positive skin test to 30μg LtSTA)					
SSL002	TIMO	13 x 14	N/A	11,329/11	88,706/88
SSL009	JAMO	17 x 17	N/A	10,430/10	47,774/45
SSL008	MIZO	19 x 15	N/A	10,055/25	38,223/96
SSL011	FAMO	23 x 24	N/A	55,262/60	66,352/72
SSL010	TIZO	19 x 17	N/A	24,301/57	54,697/129
SSL005	TISA	25 x 24	N/A	41,498/58	98,703/139

Figure 1. Proliferative Responses of Group 1 and Group 2 Subjects Expressed as Δcpm of PBMC in the Presence of PPD , LtSTA and *L.major* Antigen (LMA).



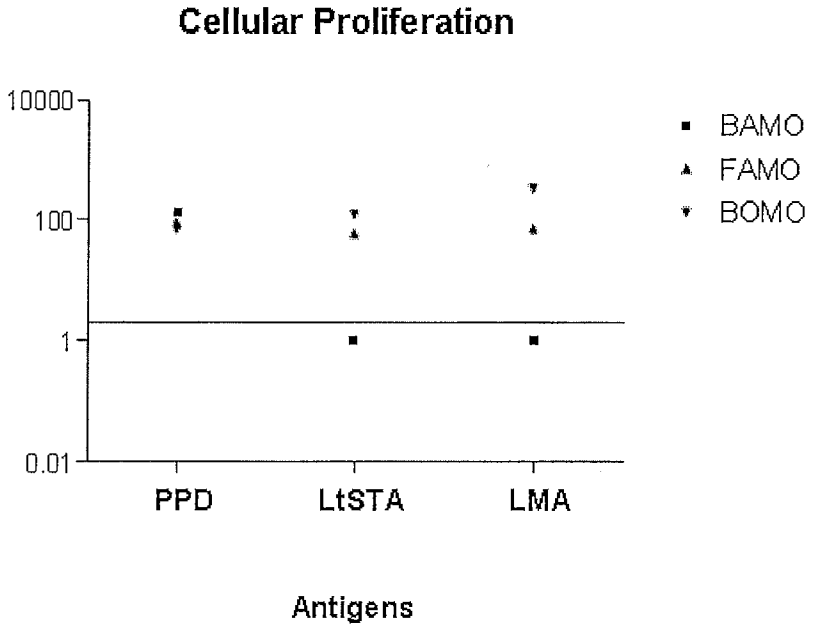
These results show that two subjects (MAHS, ABBR) out of the six individuals that had a negative skin test to 30μg LtSTA also were negative for cell proliferation to both *L.tropica* and *L.major* antigens. The level of cell stimulation of Group 1 and Group 2 subjects demonstrated the superiority of *L.major* antigen (LMA) in detecting proliferative responses in individuals with past history of disease caused by *L.major*.

In the cohort of 40 subjects without a previous history of CL subject BOMO had a positive DTH skin test to 30μg LtSTA. This subject also showed a positive cellular proliferation response to PPD, LtSTA, and LMA. This experiment was done using control PBMC samples from a matching (age, sex) subject (BAMO) who was skin test negative to 30μg LtSTA and another matching subject (FAMO) that was skin test positive to 30μg LtSTA. These results are shown in Table 9 and Figure 2.

Table 9: Results of Skin Test (30μg) and Lymphoproliferative Responses to LtSTA and *L.major* Antigen of Subject's BOMO, BAMO and FAMO

Study N°	Initials	LtSTA 30μg	LtSTA δ CPM/ Stimulation Index (SI)	*L.major* Antigen δ CPM/ Stimulation Index (SI)
No CL, Positive Skin Test to 30μg LtSTA				
SSL084	BOMO	9 X 8	31,192/ 136	85,293/ 346
No CL, Negative Skin Test to 30μg LtSTA				
SSL081	BAMO	0 X 0	102/ 1	128/ 1
CL Present, Positive Skin Test to 30μg LtSTA				
SSL011	FAMO	23 x 24	55,262/60	66,352/72

Figure 2: Proliferative Responses of BOMO Expressed as Stimulation Index (SI) of PBMC in the Presence of PPD, LtSTA and Antigen of *L.major* (LMA). PBMC of BAMO was Used as a Negative Control and Those of FAMO Were Used as a Positive Control



9.6 SAFETY OF LtSTA

No deaths, life threatening, or serious adverse events occurred to volunteers as a result of their participation in this clinical trial. The adverse events that did occur were events that are commonly observed to DTH skin test antigens. These events included local inflammation of the forearm in the immediate vicinity of positive skin tests to LtSTA, Candin®, and Trichophyton. The response included erythema and induration accompanied by mild itching and pain. No systemic allergic reactions occurred following the injection of the investigational products and no serious local reaction, such as necrosis, was observed at the site of the skin test. Three persons experienced adverse events in the cohort of twenty individuals that were involved in the titration of the four doses of LtSTA (Table 10). Doses of 10μg, 40μg, and 80μg each caused a local adverse response in one individual. These subjects were treated with local steroid (Diprosone 0.05) for several days until inflammation subsided.

One person in the cohort of forty subjects with healed cutaneous leishmaniasis within the past 24 months had a local adverse event to the 30μg dose of LtSTA (Table 11). This subject was successfully treated with parenteral steroid (Unidex 8mg) and Diprosone 0.05.

All adverse events resolved without follow up by the principal investigator or medical monitor. Tables 10 and 11 summarize the adverse events that were reported in the CRF's by the principal investigator and study staff. No adverse events were reported in the forty subjects who had no history of cutaneous leishmaniasis (Table 12).

Blood pressure, body temperature and heart rate were measured at the time of enrollment, 30 minutes before skin testing, 60 minutes after skin testing and at the time the tests were read, approximately 48 hours later. No significant changes in these measurements occurred during the course of these procedures and no evidence was found that the investigational products altered vital signs (Tables 13 - 17). Mean values for blood pressure, temperature and heart rate at the time of enrollment, before and after skin testing and 48 hours after skin testing were essentially the same. This finding indicates that the skin test procedure and the investigational products administered had no measurable adverse effect on vital signs.

Table 10: Adverse Events to Four Doses of LtSTA in Subjects with Active Cutaneous Leishmaniasis Caused by *Leishmania major*

Subject Initials	Volunteer I.D. No.	Age	Race	Sex	Active CL		LtSTA Dose		48 Hour Skin Test Reaction (Induration mm)			Adverse Events
					Yes	No	Cohort	µg	LtSTA	Saline	Placebo	
MIMA	L001	21	Ar	F	√		1	10	6.5	Ø	Ø	**No Adverse Events**
JABE	L003	49	Ar	M	√		1	10	10	Ø	Ø	**No Adverse Events**
JAWR	L004	63	Ar	F	√		1	10	14	Ø	Ø	**No Adverse Events**
JAIB	L005	22	Ar	F	√		1	10	16	Ø	Ø	**No Adverse Events**
ZAHA	L006	34	Ar	F	√		1	10	8.5	Ø	Ø	**No Adverse Events**
MASA	L002	30	Ar	F	√		2	20	16	Ø	Ø	Pain: Mild severity Erythema: Mild severity Edema: Mild severity Pruritus: Mild severity **All Adverse Events Resolved**
JAHA	L007	47	Ar	M	√		2	20	20	Ø	Ø	**No Adverse Events**
FAAB	L008	42	Ar	M	√		2	20	9.5	Ø	Ø	**No Adverse Events**
LEFA	L009	31	Ar	F	√		2	20	10	Ø	Ø	**No Adverse Events**
KARI	L010	27	Ar	M	√		2	20	16	Ø	Ø	**No Adverse Events**
HAAM	L011	32	Ar	M	√		3	40	17.5	Ø	Ø	**No Adverse Events**
GAZI	L012	42	Ar	F	√		3	40	17.5	Ø	Ø	**No Adverse Events**
TIKH	L014	28	Ar	M	√		3	40	25	Ø	Ø	Pruritus: Mild severity Erythema: Mild severity **All Adverse Events Resolved**
HAHA	L015	30	Ar	F	√		3	40	21	Ø	Ø	**No Adverse Events**
BOMO	L016	22	Ar	M	√		3	40	15	Ø	Ø	**No Adverse Events**
DHAL	L017	61	Ar	M	√		4	80	21.5	Ø	Ø	**No Adverse Events**
GAMO	L018	28	Ar	F	√		4	80	25.5	Ø	Ø	**No Adverse Events**
SLJI	L019	21	Ar	F	√		4	80	21	Ø	Ø	**No Adverse Events**
RBAB	L020	39	Ar	M	√		4	80	Ø	Ø	Ø	**No Adverse Events**
KHMA	L021	35	Ar	F	√		4	80	32	Ø	Ø	Erythema: Mild severity Pain: Mild severity Local inflammation related to administration of product **All Adverse Events Resolved**

Legend:
√ = a previous diagnosis of Leishmaniasis is indicated either negative or positive.
Ø = negative result
mm = sum of induration measuring two diameters ÷ 2

Abbreviations:
LtSTA = *Leishmania tropica* Skin Test Antigen
CL = Cutaneous Leishmaniasis
Ar = Arabic

Table 11: Adverse Events to 30μg LtSTA in Subjects with Healed Cutaneous Leishmaniasis Caused by *Leishmania major*

Subject Initials	Volunteer I.D. No.	Age	Race	Sex	CL within past 24 Months		48 Hour Skin Test Reaction (Induration mm)					Adverse Events
					Yes	No	LtSTA	Candin®	Tricho. ex	Saline	Placebo	
MINE	SSL001	39	Ar	M	√		**10.5**	11	17	Ø	Ø	**No Adverse Events**
TIMO	SSL002	48	Ar	M	√		**13.5**	12.5	5.5	Ø	Ø	**No Adverse Events**
SOZA	SSL003	42	Ar	M	√		**12.5**	12.5	13	Ø	Ø	**No Adverse Events**
FAMA	SSL004	25	Ar	F	√		**12**	10.5	20	Ø	Ø	**No Adverse Events**
TISA	SSL005	22	Ar	F	√		**24.5**	15	Ø	Ø	Ø	Local Inflammation: Mild severity **Adverse Events Resolved**
MIJA	SSL006	29	Ar	F	√		**11**	10	10	Ø	Ø	**No Adverse Events**
MIBA	SSL007	26	Ar	M	√		**19**	13	9.5	Ø	Ø	**No Adverse Events**
MIZO	SSL008	42	Ar	F	√		**17**	8.5	Ø	Ø	Ø	**No Adverse Events**
JAMO	SSL009	41	Ar	F	√		**17**	11.5	Ø	Ø	Ø	**No Adverse Events**
TIZO	SSL010	49	Ar	F	√		**18**	12	8.5	Ø	Ø	**No Adverse Events**
FAMO	SSL011	54	Ar	M	√		**23.5**	11	Ø	Ø	Ø	**No Adverse Events**
MIMOA	SSL014	33	Ar	M	√		**18**	9	12	Ø	Ø	**No Adverse Events**
MIMO	SSL015	37	Ar	M	√		**22**	18.5	7.5	Ø	Ø	**No Adverse Events**
KAMO	SSL016	21	Ar	F	√		Ø	13.5	Ø	Ø	Ø	**No Adverse Events**
MRBA	SSL017	21	Ar	F	√		**13**	9.5	Ø	Ø	Ø	**No Adverse Events**
JAHO	SSL018	22	Ar	F	√		**17**	10	12.5	Ø	Ø	**No Adverse Events**
KHRG	SSL019	59	Ar	F	√		Ø	15	Ø	Ø	Ø	**No Adverse Events**
HAHAn	SSL020	55	Ar	F	√		**15**	13	Ø	Ø	Ø	**No Adverse Events**
DHNA	SSL021	28	Ar	M	√		**21**	17	Ø	Ø	Ø	**No Adverse Events**
KHZI	SSL022	55	Ar	F	√		**9.5**	15	Ø	Ø	Ø	**No Adverse Events**
CHSI	SSL023	25	Ar	F	√		**16.5**	10	Ø	Ø	Ø	**No Adverse Events**
HAMO	SSL025	44	Ar	F	√		**11**	13.5	Ø	Ø	Ø	**No Adverse Events**
ABBR	SSL026	48	Ar	M	√		Ø	14.5	12	Ø	Ø	**No Adverse Events**
KHMO	SSL027	51	Ar	M	√		**18.5**	26	15.5	Ø	Ø	**No Adverse Events**
KHAM	SSL028	41	Ar	M	√		**15.5**	29.5	17.5	Ø	Ø	**No Adverse Events**
HAEM	SSL029	52	Ar	F	√		**14.5**	16	Ø	Ø	Ø	**No Adverse Events**
HAMAb	SSL030	56	Ar	F	√		**10**	14	Ø	Ø	Ø	**No Adverse Events**
HAHAm	SSL031	21	Ar	F	√		**6**	16	Ø	Ø	Ø	**No Adverse Events**

Legend:
√ = a previous diagnosis of Leishmaniasis is indicated either negative or positive.
Ø = negative result
mm = sum of induration measuring two diameters ÷ 2

Abbreviations:
LtSTA = *Leishmania tropica* Skin Test Antigen
Tricho Ex. = Trichophyton Extract
CL = Cutaneous Leishmaniasis
Ar = Arabic

Table 11 (continued): Adverse Events to 30μg LtSTA in Subjects with Healed Cutaneous Leishmaniasis Caused By *Leishmania major*

Subject Initials	Volunteer I.D. No.	Age	Race	Sex	CL within past 24 Months		48 Hour Skin Test Reaction (Induration mm)					Adverse Events
					Yes	No	LtSTA	Candin®	Tricho. ex	Saline	Placebo	
GAAM	SSL032	53	Ar	M	√		**13**	7	20	Ø	Ø	**No Adverse Events**
MADA	SSL034	37	Ar	F	√		**23**	14.5	17.5	Ø	Ø	**No Adverse Events**
MAHS	SSL035	57	Ar	M	√		Ø	16	Ø	Ø	Ø	**No Adverse Events**
ABEL	SSL036	51	Ar	F	√		**10**	18	Ø	Ø	Ø	**No Adverse Events**
DONA	SSL038	42	Ar	F	√		Ø	19.5	10.5	Ø	Ø	**No Adverse Events**
RIZO	SSL039	41	Ar	F	√		Ø	14	Ø	Ø	Ø	**No Adverse Events**
MIOL	SSL040	31	Ar	F	√		**18**	19	9	Ø	Ø	**No Adverse Events**
DHZI	SSL041	58	Ar	F	√		**17**	13.5	16.5	Ø	Ø	**No Adverse Events**
BEGA	SSL042	25	Ar	F	√		**7**	Ø	Ø	Ø	Ø	**No Adverse Events**
BEZA	SSL043	32	Ar	F	√		**16**	15	12	Ø	Ø	**No Adverse Events**
BEHA	SSL044	50	Ar	F	√		**21.5**	13	Ø	Ø	Ø	**No Adverse Events**
BEMO	SSL045	63	Ar	M	√		**15**	15.5	Ø	Ø	Ø	**No Adverse Events**

Legend:

√ = a previous diagnosis of Leishmaniasis is indicated either negative or positive.
Ø = negative result
mm = sum of induration measuring two diameters ÷ 2

Abbreviations:

LtSTA = *Leishmania tropica* Skin Test Antigen
Tricho Ex. = Trichophyton Extract
CL = Cutaneous Leishmaniasis
Ar = Arabic

Table 12: Adverse Events To 30μg LtSTA In Subjects With No History of Cutaneous Leishmaniasis

Subject Initials	Volunteer I.D. No.	Age	Race	Sex	CL within past 24 Months		48 Hour Skin Test Reaction (Induration mm)					Adverse Events
					Yes	No	LtSTA	Candin®	Tricho. ex	Saline	Placebo	
NCFA	SSL046	47	Ar	F		√	Ø	14.5	Ø	Ø	Ø	No Adverse Events
BOSM	SSL047	43	Ar	F		√	Ø	18.5	Ø	Ø	Ø	No Adverse Events
BOYO	SSL048	31	Ar	M		√	Ø	17.5	Ø	Ø	Ø	No Adverse Events
AKSO	SSL049	50	Ar	F		√	Ø	11	Ø	Ø	Ø	No Adverse Events
NCMO	SSL050	26	Ar	M		√	Ø	14	Ø	Ø	Ø	No Adverse Events
YONI	SSL052	25	Ar	F		√	Ø	10	Ø	Ø	Ø	No Adverse Events
JESO	SSL053	27	Ar	F		√	Ø	13	Ø	Ø	Ø	No Adverse Events
GHFA	SSL054	27	Ar	M		√	Ø	17	Ø	Ø	Ø	No Adverse Events
JAOL	SSL055	21	Ar	F		√	Ø	9.5	Ø	Ø	Ø	No Adverse Events
SANO	SSL056	21	Ar	F		√	Ø	13	Ø	Ø	Ø	No Adverse Events
ZAAB	SSL057	50	Ar	M		√	Ø	13	Ø	Ø	Ø	No Adverse Events
BOZA	SSL058	27	Ar	F		√	Ø	15.5	Ø	Ø	Ø	No Adverse Events
BOAM	SSL059	55	Ar	F		√	Ø	8	8	Ø	Ø	No Adverse Events
BALE	SSL060	25	Ar	F		√	Ø	12.5	Ø	Ø	Ø	No Adverse Events
MEIN	SSL061	27	Ar	F		√	Ø	9	Ø	Ø	Ø	No Adverse Events
MAHE	SSL062	28	Ar	M		√	Ø	13.5	Ø	Ø	Ø	No Adverse Events
NEBO	SSL064	26	Ar	M		√	Ø	13	Ø	Ø	Ø	No Adverse Events
RANA	SSL066	23	Ar	F		√	Ø	12	Ø	Ø	Ø	No Adverse Events
MBHO	SSL067	24	Ar	F		√	Ø	12.5	13	Ø	Ø	No Adverse Events
SASA	SSL068	27	Ar	F		√	Ø	16	Ø	Ø	Ø	No Adverse Events
BAME	SSL069	25	Ar	F		√	Ø	11	7	Ø	Ø	No Adverse Events

Legend:
- √ = a previous diagnosis of Leishmaniasis is indicated either negative or positive.
- Ø = negative result
- mm = sum of induration measuring two diameters ÷ 2

Abbreviations:
- LtSTA = *Leishmania tropica* Skin Test Antigen
- Tricho Ex. = Trichophyton Extract
- CL = Cutaneous Leishmaniasis
- Ar = Arabic

Table 12 (continued): Adverse Events To 30μg LtSTA In Subjects With No History of Cutaneous Leishmaniasis

Subject Initials	Volunteer I.D. No.	Age	Race	Sex	CL within past 24 Months		48 Hour Skin Test Reaction (Induration mm)					Adverse Events
					Yes	No	LtSTA	Candin®	Tricho. ex	Saline	Placebo	
BERA	SSL072	22	Ar	F		√	Ø	11	Ø	Ø	Ø	No Adverse Events
YOMO	SSL073	23	Ar	F		√	Ø	13.5	Ø	Ø	Ø	No Adverse Events
MIHA	SSL074	22	Ar	F		√	Ø	11.5	Ø	Ø	Ø	No Adverse Events
CHNA	SSL075	28	Ar	M		√	Ø	15.5	Ø	Ø	Ø	No Adverse Events
BOJA	SSL077	39	Ar	F		√	Ø	17	18.5	Ø	Ø	No Adverse Events
BOMA	SSL078	55	Ar	F		√	Ø	14.5	Ø	Ø	Ø	No Adverse Events
BODA	SSL079	38	Ar	F		√	Ø	16.5	Ø	Ø	Ø	No Adverse Events
BOSAf	SSL080	36	Ar	F		√	Ø	13	Ø	Ø	Ø	No Adverse Events
BAMO	SSL081	44	Ar	M		√	Ø	13.5	Ø	Ø	Ø	No Adverse Events
NCHO	SSL082	30	Ar	M		√	Ø	14	Ø	Ø	Ø	No Adverse Events
NCBE	SSL088	32	Ar	M		√	Ø	11	17.5	Ø	Ø	No Adverse Events
BOSE	SSL089	47	Ar	F		√	Ø	17	Ø	Ø	Ø	No Adverse Events
BOTOu	SSL090	39	Ar	F		√	Ø	11	Ø	Ø	Ø	No Adverse Events

Legend:
- √ = a previous diagnosis of Leishmaniasis is indicated either negative or positive.
- Ø = negative result
- mm = sum of induration measuring two diameters ÷ 2

Abbreviations:
- LtSTA = *Leishmania tropica* Skin Test Antigen
- Tricho Ex. = Trichophyton Extract
- CL = Cutaneous Leishmaniasis
- Ar = Arabic

Table 13: Vital Signs of Study Participants Involved in the Titration of LtSTA: Measured at Enrollment, 30 Minutes Before Skin Testing, 60 Minutes after Skin Testing, and 48 Hours after Skin Testing

Subject Initials	Study I.D. No.	At Enrollment				30 Minutes Before Skin Testing				60 Minutes after Skin Testing				48 Hours After Skin Testing			
		Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.
		Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic		
MIMA	L001	110	70	98.9	80	N/A	N/A	N/A	N/A	110	70	98.7	80	110	70	99.2	84
MASA	L002	130	80	97.9	68	120	80	99.3	64	110	75	98.6	68	110	80	99.3	66
JABE	L003	110	60	95.7	80	110	70	98.0	72	105	65	97.7	72	120	80	97.5	64
JAWR	L004	160	80	99.0	80	150	70	97.8	92	130	65	98.3	64	110	60	98.0	64
JAIB	L005	100	80	98.2	64	110	60	97.7	72	105	60	97.3	76	110	60	97.9	68
ZAHA	L006	120	80	98.8	80	120	70	98.7	76	115	70	97.9	84	110	70	98.3	76
JAHA	L007	130	70	97.1	80	130	70	97.1	80	120	70	96.0	68	130	80	98.2	68
FAAB	L008	120	70	97.1	60	120	75	98.0	64	110	70	97.5	60	105	60	97.5	68
LEFA	L009	100	60	99.6	65	110	70	98.9	80	100	65	98.8	72	105	60	99.0	72
KARI	L010	110	70	97.9	68	130	80	96.7	68	120	80	96.9	68	110	70	98.3	64
HAAM	L011	120	80	98.5	70	110	70	97.4	56	130	70	97.7	56	110	80	98.5	64
GAZI	L012	110	70	98.3	60	100	60	98.1	64	100	60	98.1	60	100	70	98.5	56
TIKH	L014	110	80	97.9	64	100	70	97.8	64	105	60	97.5	56	100	60	98.2	68
HAHA	L015	130	80	97.5	88	120	70	98.2	100	120	70	98.5	92	120	80	98.5	92
BOMO	L016	110	80	97.2	56	110	80	97.2	56	110	70	98.4	56	120	70	98.8	68
DHAL	L017	140	90	98.6	80	120	80	98.3	80	110	70	98.2	80	130	80	98.2	68
GAMO	L018	120	80	97.5	80	110	70	99.6	84	100	60	99.6	80	100	60	98.7	72
SLJI	L019	100	70	97.0	80	100	70	97.3	68	100	60	98.2	68	95	60	97.2	64
RBAB	L020	130	70	98.2	72	110	60	98.0	64	120	80	98.6	64	130	90	98.1	60
KHMA	L021	100	60	98.6	68	100	50	98.8	60	110	60	98.3	60	100	70	98.0	68
Sum		2360.0	1480.0	1959.5	1443.0	2180.0	1325.0	1862.9	1364.0	2230.0	1350.0	1960.8	1384.0	2225.0	1410.0	1965.9	1374.0
Mean		118.0	74.0	98.0	72.2	114.7	69.7	98.0	71.8	111.5	67.5	98.0	69.2	111.3	70.5	98.3	68.7

Table 14: Vital Signs of Study Participants with No History of Cutaneous Leishmaniasis: Measured at Enrollment, 30 Minutes Before Skin Testing, 60 Minutes After Skin Testing, and 48 Hours After Skin Testing

Subject Initials	Study I.D. No.	At Enrollment				30 Minutes Before Skin Testing				60 minutes After Skin Testing				48 Hours After Skin Testing			
		Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.
		Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic		
DHZI	SSL041	130	80	98.3	68	110	70	99.1	96	120	70	98.5	66	120	70	98.5	70
BEGA	SSL042	100	60	97.5	72	100	60	97.7	80	90	50	98.2	80	90	50	99.1	78
BEZA	SSL043	100	60	98.1	64	120	80	99.1	72	120	80	98.7	68	120	70	99.0	60
BEHA	SSL044	120	80	97.6	76	120	80	98.7	76	120	80	98.1	68	120	70	98.0	64
BEMO	SSL045	140	90	97.0	76	140	85	98.8	80	140	80	98.1	68	120	75	98.1	62
NCFA	SSL046	110	60	98.5	68	110	60	97.9	72	120	80	98.4	70	140	70	98.7	84
BOSM	SSL047	110	80	97.4	76	110	70	97.9	62	110	55	98.1	66	120	70	99.0	76
BOYO	SSL048	110	70	98.1	60	130	70	98.2	64	110	65	98.3	56	120	70	98.5	82
AKSO	SSL049	130	80	96.7	56	120	70	97.3	52	140	90	97.7	56	120	85	97.9	58
NCMO	SSL050	120	70	97.6	64	120	80	98.1	68	120	70	98.1	62	120	80	98.5	68
YONI	SSL052	110	70	99.4	80	100	50	98.6	68	100	55	98.6	60	100	60	98.7	72
JESO	SSL053	110	70	98.5	76	130	80	98.3	84	100	65	98.0	74	110	70	97.7	72
GHFA	SSL054	110	60	98.4	76	95	50	97.7	68	95	55	97.9	66	90	65	98.4	72
JAOL	SSL055	100	60	99.5	80	100	65	97.8	60	100	65	98.3	56	100	60	98.0	58
SANO	SSL056	110	70	98.2	72	110	60	97.5	60	100	55	97.0	56	110	70	97.4	60
ZAAB	SSL057	130	70	98.3	80	130	70	98.3	80	120	60	98.7	72	120	80	98.3	74
BOZA	SSL058	120	70	97.0	80	100	60	97.0	76	100	60	98.3	80	100	60	96.6	74
BOAM	SSL059	100	60	97.3	64	100	60	98.3	76	100	60	98.7	72	100	60	98.7	76
BALE	SSL060	95	60	98.3	72	110	60	98.5	76	100	60	98.6	56	100	60	98.7	64
MEIN	SSL061	100	70	97.5	60	95	55	97.9	54	100	60	96.8	60	100	60	97.3	66
MAHE	SSL062	120	70	98.0	76	110	70	98.4	68	110	70	96.8	88	120	80	98.4	82
NEBO	SSL064	110	60	97.2	64	100	55	96.5	65	110	60	96.5	66	100	60	97.0	64
RANA	SSL066	110	70	98.6	80	110	70	98.3	76	105	65	98.5	68	120	70	98.5	78
MBHO	SSL067	120	70	98.2	68	110	70	98.2	76	110	70	98.2	72	110	60	98.9	98
SASA	SSL068	90	60	98.5	72	90	60	97.6	74	95	55	98.6	68	90	55	97.8	66
Sum		2805.0	1720.0	2449.7	1780.0	2770.0	1660.0	2451.7	1783.0	2735.0	1635.0	2451.7	1674.0	2760.0	1680.0	2455.7	1778.0
Mean		112.2	68.8	98.0	71.2	110.8	66.4	98.1	71.3	109.4	65.4	98.1	67.0	110.4	67.2	98.2	71.1

TABLE 14 (continued): Vital Signs of Study Participants with No History of Cutaneous Leishmaniasis: Measured at Enrollment, 30 Minutes Before Skin Testing, 60 Minutes After Skin Testing, and 48 Hours After Skin Testing

Subject Initials	Study I.D. No.	At Enrollment				30 Minutes Before Skin Testing				60 minutes After Skin Testing				48 Hours After Skin Testing			
		Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.
		Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic		
BAME	SSL069	120	70	97.5	76	95	50	97.9	64	100	60	97.1	70	110	60	98.9	66
BERA	SSL072	100	60	98.3	60	90	60	98.5	64	110	55	98.5	70	100	60	98.2	72
YOMO	SSL073	100	60	98.8	64	110	70	98.8	60	100	60	99.0	64	100	65	99.1	66
MIHA	SSL074	100	70	97.2	68	100	60	97.9	64	100	60	98.5	70	120	70	98.5	78
CHNA	SSL075	110	70	98.2	56	100	60	98.0	60	100	60	98.2	54	100	60	98.7	54
OUWR	SSL076	120	60	97.8	76	110	70	98.4	80	110	60	96.2	74	120	70	98.0	72
BOJA	SSL077	120	80	98.7	64	130	80	98.5	70	120	80	98.6	66	120	70	97.5	72
BOMA	SSL078	100	60	97.0	68	95	50	97.4	70	100	70	97.6	60	90	50	97.0	60
BODA	SSL079	120	80	97.9	76	140	80	98.5	66	130	80	98.0	66	120	70	98.7	68
BOSAf	SSL080	120	80	98.4	84	160	95	97.6	82	150	90	97.6	80	140	90	98.1	84
BAMO	SSL081	130	90	98.0	80	140	95	98.3	82	130	90	98.1	82	130	80	97.4	72
NCHO	SSL082	120	80	98.3	60	130	60	97.7	60	120	60	97.2	66	125	65	97.2	58
BODH	SSL083	130	80	97.9	72	160	90	98.0	72	130	80	97.4	62	120	80	97.0	72
BOMO	SSL084	130	80	97.6	68	130	75	98.9	72	140	80	97.9	68	140	80	97.3	68
NCMH	SSL085	160	90	97.6	60	150	90	96.7	62	120	80	98.0	62	140	80	97.8	64
NCAI	SSL086	120	80	98.2	80	130	80	98.7	62	140	70	98.7	62	120	60	98.7	68
BOAL	SSL087	130	80	97.1	68	130	90	97.9	80	130	95	98.0	76	140	80	98.0	80
NCBE	SSL088	130	80	90.7	64	150	70	98.3	74	120	70	98.8	72	130	70	98.2	68
	Sum	2160.0	1350.0	1755.2	1244.0	2250.0	1325.0	1766.0	1244.0	2150.0	1300.0	1763.4	1224.0	2165.0	1260.0	1764.3	1242.0
	Mean	120.0	75.0	97.5	69.1	125.0	73.6	98.1	69.1	119.4	72.2	98.0	68.0	120.3	70.0	98.0	69.0

Table 15: Vital Signs of Study Participants with a History of Cutaneous Leishmaniasis within the Past 24 Months: Measured at Enrollment, 30 Minutes Before Skin Testing, 60 Minutes After Skin Testing, and 48 Hours After Skin Testing

Subject Initials	Study I.D. No.	At Enrollment				30 Minutes Before Skin Testing				60 minutes After Skin Testing				48 Hours After Skin Testing			
		Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.
		Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic		
MINE	SSL001	120	80	97.6	64	130	80	96.3	68	130	75	97.1	60	140	85	96.3	60
TIMO	SSL002	110	70	97.6	60	105	70	98.7	96	100	70	98.1	72	110	80	98.5	66
SOZA	SSL003	110	80	97.8	68	110	80	97.8	68	100	80	98.0	60	100	70	97.9	60
FAMA	SSL004	130	70	98.7	76	120	70	97.6	76	130	70	98.5	80	130	70	99.0	66
TISA	SSL005	130	70	98.5	60	120	65	99.4	72	110	60	97.9	72	115	70	98.3	66
MIJA	SSL006	110	80	98.7	84	120	80	98.7	84	120	80	100.0	84	130	80	99.1	80
MIBA	SSL007	110	80	98.6	68	120	70	98.6	80	120	60	98.8	80	120	40	98.5	70
MIZO	SSL008	110	70	97.2	80	110	70	98.7	96	120	70	98.1	72	120	70	97.6	70
JAMO	SSL009	130	80	98.0	68	120	80	97.9	72	110	70	99.0	72	120	75	99.1	66
TIZO	SSL010	100	60	98.2	56	125	70	99.1	68	130	80	98.5	68	120	80	98.2	62
FAMO	SSL011	120	80	97.0	72	110	80	97.7	68	120	75	97.9	72	120	85	97.8	80
MIMOA	SSL014	110	80	98.2	60	100	60	98.7	64	105	70	97.9	60	100	65	97.5	58
MIMO	SSL015	130	90	98.8	88	130	85	94.5	96	120	60	95.1	80	110	80	98.5	66
KAMO	SSL016	110	70	98.7	76	110	55	98.8	80	110	60	98.3	88	120	80	98.6	62
MRBA	SSL017	120	60	97.5	72	120	70	98.0	68	120	80	99.1	60	120	70	98.6	70
JAHO	SSL018	110	70	98.8	68	120	70	98.6	68	110	65	98.0	68	130	80	99.2	76
KHRG	SSL019	130	80	97.0	72	180	100	98.4	80	190	100	98.3	84	120	80	97.9	66
HAHAn	SSL020	130	80	97.9	76	150	95	100.0	76	150	90	99.4	72	140	80	98.5	76
DHNA	SSL021	120	70	98.0	72	110	70	97.1	72	100	70	95.5	64	100	75	94.8	66
KHZI	SSL022	140	80	97.8	76	160	100	98.1	84	150	90	98.4	80	150	85	98.3	68
CHSI	SSL023	120	80	97.9	76	120	80	99.3	72	120	80	100.1	76	130	70	99.8	66
HAMO	SSL025	140	80	98.0	68	130	90	98.5	80	130	90	99.4	64	130	80	99.3	66
Sum		2640.0	1660.0	2156.5	1560.0	2720.0	1690.0	2160.5	1688.0	2695.0	1645.0	2161.4	0588.0	2675.0	1650.0	2161.3	1486.0
Mean		120.0	75.5	98.0	70.9	123.6	76.8	98.2	76.7	122.5	74.8	98.2	72.2	121.6	75.0	98.2	67.5

Table 15 (continued): Vital Signs of Study Participants with a History of Cutaneous Leishmaniasis within the Past 24 Months: Measured at Enrollment, 30 Minutes Before Skin Testing, 60 Minutes After Skin Testing, and 48 Hours After Skin Testing

Subject Initials	Study I.D. No.	At Enrollment				30 Minutes Before Skin Testing				60 minutes After Skin Testing				48 Hours After Skin Testing			
		Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.
		Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic		
ABBR	SSL026	140	90	98.2	74	140	80	97.7	84	130	80	97.8	72	120	80	97.6	82
KHMO	SSL027	130	80	98.5	72	130	65	99.0	84	135	75	99.1	84	125	70	99.3	62
HAMAb	SSL030	150	100	98.5	80	180	110	99.0	80	180	110	99.1	84	150	90	98.7	74
HAHAm	SSL031	100	60	97.2	60	130	80	97.9	72	100	70	98.5	68	110	70	98.5	68
GAAM	SSL032	120	80	97.8	72	140	90	97.6	92	110	80	97.5	84	140	80	97.6	74
MADA	SSL034	120	80	97.1	72	130	80	98.8	76	130	70	98.7	68	140	70	99.0	78
MAHS	SSL035	140	90	97.7	72	140	100	97.6	72	140	100	97.8	68	130	85	97.7	72
ABEL	SSL036	110	10	97.8	60	110	60	98.7	60	100	60	97.8	64	95	55	97.5	60
DONA	SSL038	130	80	98.0	65	120	70	97.2	64	140	80	98.1	72	130	70	98.7	62
RIZO	SSL039	120	80	98.2	68	140	80	98.9	72	140	80	98.2	76	150	90	98.2	82
MIOL	SSL040	100	70	97.9	92	120	80	99.9	72	100	70	99.4	76	110	70	99.1	82
Sum		1360.0	820.0	1076.9	787.0	1480.0	895.0	1082.3	828.0	1405.0	875.0	1082.0	816.0	1400.0	830.0	1081.9	796.0
Mean		123.6	74.5	97.9	71.5	134.5	81.4	98.4	75.3	127.7	79.5	98.4	74.2	127.3	75.5	98.4	72.4

Table 16: Vital Signs of Study Participants Tested with 50µg LtSTA: Measured at Enrollment, Before Skin Testing, 60 Minutes After Skin Testing, and 48 Hours after Skin Testing

Subject Initials	Study I.D. No.	At Enrollment				Before Skin Testing				60 Minutes After Skin Testing				48 Hours After Skin Testing			
		Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.
		Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic		
KHRG	SSL034	130	70	97.9	84	130	70	97.9	84	120	70	98.6	82	150	90	98.0	90
DONA	SSL032	100	60	98.7	80	100	60	98.7	80	110	70	98.7	68	110	70	97.4	67
MAHS	SSL031	120	80	98.8	60	120	80	98.8	60	110	60	98.8	78	130	80	97.5	80
RIZO	SSL033	120	70	99.2	88	120	70	99.2	88	120	70	99.6	92	140	80	98.4	122
ABBR	SSL036	120	70	98.6	68	120	70	98.6	68	120	70	98.5	92	130	80	97.7	87
KAMO	SSL035	100	60	98.9	76	100	60	98.9	76	105	60	99.1	76	100	60	97.9	65
Sum		690.0	41.0	592.1	456.0	690.0	41.0	592.1	456.0	685.0	400.0	593.3	488.0	760.0	460.0	586.9	511.0
Mean		115.0	68.3	98.7	76.0	115.0	68.3	98.7	76.0	114.2	66.7	98.9	81.3	126.7	76.7	97.8	85.2

Table 17: Vital Signs of Study Participants Used as Controls for Subjects Listed in Table 16: Measured at Enrollment, Before Skin Testing, 60 Minutes After Skin Testing, and 48 Hours After Skin Testing

Subject Initials	Study I.D. No.	At Enrollment				Before Skin Testing				60 Minutes After Skin Testing				48 Hours After Skin Testing			
		Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.	Blood Pressure		Temp. °F	Heart Rate beats/min.
		Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic			Systemic	Diastolic		
TIMO	SSL002	110	70	97.6	60	105	70	98.7	96	100	70	98.1	72	110	80	98.5	66
TISA	SSL005	130	70	98.5	60	120	65	99.4	72	110	60	97.9	72	115	70	98.3	66
MIZO	SSL008	110	70	97.2	80	110	70	98.7	96	120	70	98.1	72	120	70	97.6	70
JAMO	SSL009	130	80	98.0	68	120	80	97.9	72	110	70	99.0	72	120	75	99.1	66
TIZO	SSL010	100	60	98.2	56	125	70	99.1	68	130	80	98.5	68	120	80	98.2	62
FAMO	SSL011	120	80	97.0	72	110	80	97.7	68	120	75	97.9	72	120	85	97.8	80
Sum		700.0	430.0	586.5	396.0	690.0	435.0	591.5	472.0	690.0	425.0	589.5	428.0	705.0	460.0	589.5	410.0
Mean		116.7	71.7	97.8	66.0	115.0	72.5	98.6	78.7	115.0	70.8	98.3	71.3	117.5	76.7	98.3	68.3

10. STATISTICAL ANALYSIS OF DATA

10.1 BACKGROUND

The Tunisia 2007 study was conducted to evaluate the sensitivity and specificity of LtSTA in a *Leishmania major* population in Tunisia. The study was designed to show that the population sensitivity and specificity to LtSTA are greater than minimally acceptable levels of 80% and 85%, respectively, i.e. the null and alternative hypotheses are:

1) For sensitivity

H_0 : Sensitivity ≥ 0.80 vs. H_A : Sensitivity < 0.80

2) For specificity

H_0 : Specificity ≥ 0.85 vs. H_A : Specificity < 0.85

Sensitivity is defined as the proportion of the population with a history of cutaneous leishmaniasis caused by *L.major* that have a positive skin test to LtSTA. A false negative is defined as a person with the disease who does not test positive LtSTA.

Specificity is defined as the proportion of the population with no history of cutaneous leishmaniasis caused by *L.major* that have a negative skin test to LtSTA. A false positive is defined as a person without the disease who tests positive with LtSTA.

10.2 DATA FOR SENSITIVITY

A total of 40 subjects with a history of cutaneous leishmaniasis, were skin tested with 30µg LtSTA. Out of 40 subjects, 34 subjects had positive skin tests to 30µg LtSTA. Six subjects who were negative to 30µg LtSTA were skin tested with 50µg LtSTA. Out of 6 subjects, 6 subjects had positive skin tests to 50µg LtSTA.

10.3 DATA FOR SPECIFICITY

A total of 40 subjects with no history of cutaneous leishmaniasis were skin tested with 30µg LtSTA. Out of 40 subjects, 39 subjects had negative skin tests to LtSTA. A 50µg dose of LtSTA was not tested in this cohort.

10.4 RESULTS

For this analysis Software STATA Version 10 was used. To confirm whether sensitivity could be considered at least 80% and specificity at least 85%, the 95% one-sided confidence intervals (CI) were justified. Sensitivity and specificity results for the 30µg dose of LtSTA are shown in Tables A and B, respectively. Sensitivity for the 50µg dose of LtSTA is show in Table C.

Table A: For Sensitivity for 30µg LtSTA

N	Positive	Observed Sensitivity (%)	One-Sided 95% Lower CI (%)
40	34	85.0	72.5

Table B: For Specificity for 30µg LtSTA

N	Negative	Observed Specificity (%)	One-Sided 95% Lower CI (%)
40	39	97.5	86.8

Table C: For Sensitivity for 50µg LtSTA

N	Positive	Observed Sensitivity (%)	One-Sided 95% Lower CI (%)
40	40	100	92.8*

*This value is based on the assumption that the 34 subjects (Table A) who were skin test positive to 30µg LtSTA would be skin test positive to 50µg LtSTA. Therefore, the observed sensitivity would have been 100% (40/40) with a one-sided lower confidence of 92.8%.

10.5 CONCLUSION

Based on the data of this study, we conclude with 95% confidence that the sensitivity and specificity to 30µg LtSTA in an *L.major* population are at least 73% and 87%, respectively. The sensitivity to 50µg LtSTA in the same population is at least 93%.

11. COMMENTS AND CONCLUSIONS

This phase II clinical trial was conducted as part of a continuing effort to develop a standardized skin test antigen with appropriate sensitivity and specificity to detect prior exposure to *Leishmania major*. Leishmaniasis is a world-wide medical problem that affects millions of people in over 80 different countries. The disease occurs in several different clinical forms and is caused by a number of different species. Table 18 below lists five different species of *Leishmania* as causal agents of visceral disease; six different species that are responsible for cutaneous disease in Africa, Asia, India, Middle East; twelve species that cause cutaneous leishmaniasis in North, Central and South America; and two species that invade the mucosa in both the Old World (Africa) and New World (Central and South America).

Table 18. Clinical syndromes and geographic distribution of *Leishmania* spp. (13)

Clinical Syndrome	*Leishmania* species	Location
Visceral leishmaniasis		
Kala-azar: generalized involvement of the reticuloendothelial system (spleen, bone marrow, liver, etc.)	*L. donovani*	Indian subcontinent, north and east China, Pakistan, Nepal, east Africa
	L. infantum	Middle East, Mediterranean littoral, Balkans, central and south-west Asia, north and north-west China, north and sub-Saharan Africa
	L. chagasi	Latin America
	L. amazonensis	Brazil (Bahia State)
	L. tropica	Mediterranean littoral, Middle East, north Africa, Pakistan, India, south-west Asia
Post-kala-azar dermal leishmaniasis	*L. donovani*	Indian subcontinent, north and east China, Pakistan, Nepal, east Africa
Old World cutaneous leishmaniasis		
Single or limited number of skin lesions	*L. major* "wet ulcer"	Middle East, north-west China, north-west India, Pakistan, Africa, south-west Asia
	L. tropica "dry ulcer"	Mediterranean littoral, Middle East, north Africa, Pakistan, India, south-west Asia
	L. aethiopica	Ethiopian highlands, Kenya, Yemen
	L. infantum (rare)	Mediterranean basin
	L. donovani (rare)	Sudan, east Africa
Diffuse cutaneous leishmaniasis	*L. aethipica*	Ethiopian highlands, Kenya, Yemen
New World cutaneous leishmaniasis		
Single or limited number of skin lesions	*L. Mexicana*	Central and South America, Texas, Mexico
	L. amazonensis	Amazon basin, neighboring areas, Bahia and other states in Brazil
	L. (viannia) braziliensis	Multiple areas of Central and South America
	L guyanensis.	Guyanas, Surinam, northern Amazon basin
	L. peruviana	Peru (western Andes), Argentinean highlands
	L. panamensis	Panama, Costa Rica, Columbia
	L. Pifanoi	Venezuela
	L. garmhami	Venezuela
	L. venezuelensis	Venezuela
	L. columbiensis	Colombia and Panama
	L. chagasi	Central and South America
Diffuse cutaneous leishmaniasis	*L. amazonensis*	Amazon basin, neighboring areas, Bahia and other states in Brazil
	L. pifanoi	Venezuela
	L. Mexicana	Mexico, Central America
Mucosal leishmaniasis (espundia)	*L. braziliensis* (New World)	Central and South America
	L. aethiopica (Old World)	Ethiopian highlands, Kenya, Yemen

Whole cell suspensions of *Leishmania* promastigotes, as well as soluble preparations of promastigotes have been used as skin test antigens for both diagnostic and epidemiologic purposes. In some instances, the antigen has been prepared from locally isolated strains of the parasite without consideration for appropriate controls or manufacturing methods. This has resulted in published information that is difficult to evaluate in terms of the reliability of the skin test in detecting sensitivity to the homologous agent, or to cross-reacting species. As a rule, maximum sensitivity and specificity of a skin test antigen occur when the antigen is prepared from the same agent that it is intended to detect. However, studies have shown that antigen made from one *Leishmania* species can be used to detect exposure to other species of the parasite.

Species specific antigens of *L.major* have been demonstrated by testing purified portions of *L.major* promastigotes in guinea pigs sensitized to *L.major*, *L.tropica*, and *L.infantum* (14). These experiments showed that purified antigen of *L.major* reacted in *L.major* sensitized animals, but did not react in animals sensitized to *L.tropica* or *L.infantum*. Specific antigens undoubtedly occur in all *Leishmania* species; however, it is impractical to consider developing species-specific skin test antigens based on current pharmaceutical standards. As an alternative approach, standardized skin test products could be prepared from antigenically dominant species which share cross reaction components with other species. The results of the present study demonstrate the utility of this approach for *L.tropica* and *L.major*.

In a study by Abramson et al. (15) the use of soluble antigen made from *L.chagasi* evoked a positive skin test response in 91% of cases of American visceral leishmaniasis, whereas antigen of *L.infantum* was reactive in 71% of the same population. In cases of cutaneous leishmaniasis, skin test antigen made from *L.braziliensis*, *L.guyanensis*, and *L.mexicana amazonensis* evoked positive skin tests in 100% of subjects, while *L.major* antigen was positive in only 19%. Based on these findings, a multivalent *Leishmania* skin test antigen should be prepared from representative dominant antigen species from both the Old and New World groups.

Akuffo et al. (16) evaluated skin test reactivity to two commercial preparations of *Leishmania major* skin test antigen in leishmaniasis patients from Ethiopia (*L.aethipica*) and Nicaragua (probably *L.braziliensis* complex). The purpose of their investigation was to see if *L.major* antigen would detect sensitivity to *L.aethiopica* and *L.braziliensis*. One preparation was superior in identifying the majority (83-90%) of confirmed cases of local cutaneous leishmaniasis (LCL) from Ethiopia. The skin test antigen, which performed less well (showing a positive result in only 50% of the LCL patients) showed promise when used to test active and healed cases of a leishmaniasis in Nicaragua (positive result in 92% of the active and healed patients). The author's concluded that cross-reacting *Leishmania* species may be considered for use in the preparation of standardized leishmanin antigen; however, differences in the methods of preparation may affect antigenicity, and thus its efficacy in detecting different forms of leishmaniasis in different geographic areas.

Agwale et al. (11) found different outcomes when skin test antigens prepared from promastigotes of either *Leishmania major* or *L.amazonensis*, or pooled *L.mexicana*, *L.amazonensis* and *L.guyanensis* were tested in cutaneous leishmaniasis (CL) patients and healthy subjects living in two endemic foci in Nigeria. Their study was designed to: (1) provide insights into whether cross-species leishmanin, such as that prepared from New World *Leishmania* could be used to detect cases of Old World leishmanial infection, and (2) compare the results with *L.major*-derived leishmanin. The overall skin positivity in individuals from Keana tested with the cross-species leishmanin was 28.7% (27/94); the positivity rate in the subjects from Kanana tested with the same leishmanin was 54.4% (6/11). Lower positivity values were obtained when *L.major* (12.5%; 11/88) or *L.amazonensis* (15.8%; 9/57) was tested as antigen in comparable populations. Pooled antigen identified most of the subjects (13/14; 92.9%) with active or healed CL, and the maximum reaction sizes were found among positive subjects in this group. No healthy controls (10 total) showed specific DTH response.

In the present study, *L.tropica* soluble antigen (LtSTA) was employed to detect sensitivity to *L.major*. The decision to conduct the trial in an *L.major* endemic area was based on laboratory data from guinea pigs which showed DTH cross-reactivity between the two species. The hypothesis that *L.tropica* antigen could detect prior exposure to *L.major* in humans was proven valid in subjects with active and healed CL caused by *L.major*. Dose-response testing of persons with active CL with LtSTA concentrations of 10, 20, 40, and 80μg demonstrated 100% reactivity. As anticipated, the size of the DTH skin test response increased with the corresponding increase in dose; however, the test was positive at all four dose levels. From these data, a dose of 30μg was selected to evaluate the sensitivity of LtSTA in subjects with healed *L.major* CL. This work revealed that 85% of persons with a history of CL within the past 24 months were skin test positive to LtSTA. Conversely, testing LtSTA in persons residing in an endemic area for *L.major*, but without histories of CL, demonstrated a high level of product specificity. In this population LtSTA did not elicit positive skin tests in 97% of the subjects tested. In a follow-up investigation, six subjects with histories of healed CL who failed to react to the 30μg dose of LtSTA were skin tested with a 50μg dose. All six subjects had positive skin tests to the 50μg dose. This finding confirmed that sensitivity to cross-reacting components also was present in these individuals, but required a higher concentration of LtSTA to evoke a measureable induration response.

The reactivity pattern observed with ascending doses of LtSTA between 10 and 80μg in subjects with active CL demonstrated the levels at which cross-reactivity occurs between *L.tropica* and *L.major*, i.e. in persons with current disease where sensitivity might be most pronounced, a relatively small dose (10μg) of the product was capable of evoking a positive DTH response. With the passing of time, sensitivity decreased in some individuals as reflected in the skin test patterns of the six subjects who were negative to the 30μg dose, but positive to the 50μg dose.

One of the primary objectives of this phase II clinical trial was to identify a dose, or dose range of LtSTA that would provide levels of sensitivity and specificity that were acceptable for a diagnostic skin test antigen. The observed sensitivity of LtSTA in a population of forty (40) adults with healed CL caused by *L.major* was 85%. Based on lymphocyte proliferation experiments with both *L.major* and *L.tropica* antigen (LtSTA), the two subjects in this cohort that were skin test negative to LtSTA may have lacked the ability to mount a normal DTH response to a *Leishmania* skin test antigen. With this consideration, the observed sensitivity of the product would have increased to 90% (36/40) at a dose of 30μg. The successful outcome of dose range of 25 to 50μg of *Leishmania* antigen (based on protein content) was reported by Reed et al. (17) in studies of American visceral leishmaniasis caused by *L.chagasi.* At these concentrations, their skin test antigen made from *L.chagasi* promastigotes evoked positive skin tests in 95-100% of study participants. These investigators also reported that 25 to 50μg of antigen made from *L.mexicana amazonensis* produced positive skin tests in 82% of the subjects with cutaneous leishmaniasis.

The data obtained in this investigation support the continued development of LtSTA as a diagnostic skin test antigen for leishmaniasis caused by *L.major.* Additional studies with the product in persons infected with other species of the parasite are needed to evaluate the safety and efficacy of LtSTA in populations other than those infected with *L.major.* Future studies with LtSTA will be conducted at dose levels of 15, 30, and 50μg in naïve subjects to evaluate the sensitizing effects of repeat skin testing with these doses. The ability to retest persons with LtSTA as they return from endemic areas for *L.major* is essential for the product to be useful as a screening test for leishmanial disease. The extent to which repeat skin testing results in negative to positive conversion must be established for LtSTA to be a reliable tool in detecting exposure to *Leishmania* parasites.

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Attachment 2

Protocol (LtSTA-08 Rev 3) for Phase IIb Clinical Trial.

BB-IND 11822
Investigational Product: *Leishmania tropica* Skin Test Antigen (LtSTA)

CLINICAL PROTOCOL

Title: A Blinded, Placebo Controlled Study Evaluating Safety, False-Positive Reactions and Sensitizing Properties of 15µg, 30µg and 50µg Intracutaneous Doses of *Leishmania tropica* Skin Test Antigen (LtSTA) In Adult Volunteers Without a History of Exposure to *Leishmania spp.*

Phase of Development: II
Study # LtSTA-08 Revision 03
July 21, 2008

This study will be performed in compliance with good clinical practice (GCP), including the archiving of essential documents.

Sponsor:

07-21-08

H. S. Nielsen, Jr., Ph.D. **Date**
President
Allermed Laboratories, Inc.

Principal Investigator:

7/21/08

Donald Brandon, M.D. **Date**
California Research Foundation
(single study site)
2800 Third Ave
San Diego, CA 92103-6204

BB-IND 11822
Investigational Product: *Leishmania tropica* Skin Test Antigen (LtSTA)

CLINICAL PROTOCOL

Title: A Blinded, Placebo Controlled Study Evaluating Safety, False-Positive Reactions and Sensitizing Properties of 15µg, 30µg and 50µg Intracutaneous Doses of *Leishmania tropica* Skin Test Antigen (LtSTA) In Adult Volunteers Without a History of Exposure to *Leishmania spp.*

Phase of Development: II
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This study will be performed in compliance with good clinical practice (GCP), including the archiving of essential documents.

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President
Allermed Laboratories, Inc.

Principal Investigator:

7/21/08

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TABLE OF CONTENTS

1. GENERAL INFORMATION

USAMMDA Contract Number:	DAMD 17-00-C-0030
IND Number:	BB-IND 11822
Study Number:	LtSTA-08
Sponsor:	Allermed Laboratories, Inc. 7203 Convoy Ct. San Diego, CA 92111 Phone: 858-292-1060 Attn: Stewart Nielsen, Ph.D.
Principal Investigator:	Donald Brandon, M.D. California Research Foundation 2800 Third Ave, San Diego, CA 92103-6204 Phone: (619) 291-2321
Medical Monitor:	Bruce Sahba, M.D., F.A.C.G. 1855 First Ave Suite 200B San Diego, CA 92101 619-702-2100
Clinical Study Site:	California Research Foundation 2800 Third Ave, San Diego, CA 92103-6204 Phone: (619) 291-2321
Federal-Wide Assurance Number of Clinical Study Site:	FWA00006704
Clinical Laboratory:	Sharp Memorial Hospital laboratory 7901 Frost Street San Diego, CA. (858)939-3650
Study Duration:	6 months
Anticipated Start Date:	August, 2008
Required Number of Volunteers:	30
Required Number of Alternate Volunteers:	6
Total Number of Volunteers:	36
IRB:	Biomed Research Institute of America 3110 Camino del Rio South, Suite A215 San Diego, CA 92108 Phone: (619) 282-9997 Fax: (619)282-9998
Federal-wide Assurance Number of Sponsor:	FWA00011067
Human Research Protection Office (HRPO):	Contact: 504 Scott Street, Fort Detrick, MD 21702-5012 Phone: (301) 619-6239

2. SYNOPSIS

Sponsor: Allermed Laboratories, Inc **Name of Finished Product:** *Leishmania tropica* Skin Test Antigen (LtSTA) **Active Ingredient:** *Leishmania tropica* Promastigote Lysate	BB IND 11822 LtSTA-08, Rev. 03 San Diego	
Title of Study: A Blinded, Placebo Controlled Study Evaluating Safety, False-Positive Reactions and Sensitizing Properties of 15μg, 30μg and 50μg Intracutaneous Doses of *Leishmania tropica* Skin Test Antigen (LtSTA) in Adult Volunteers Without a History of Exposure to *Leishmania spp.*		
Investigator(s): Dr. Donald Brandon, M.D.		
Study Center: California Research Foundation 2800 Third Ave, San Diego, CA 92103-6204		
Publication (reference): None		
Study Period: 6 months Anticipated date of first enrollment: 05/08 Anticipated date of completion: 11/08	Phase of Development: II	
Objectives: (1) to evaluate the safety of 15μg, 30μg and 50μg/0.1mL doses of LtSTA in healthy adult volunteers who have had no known previous exposure to *Leishmania* parasites; (2) to provide information on the occurrence of false-positive skin tests to LtSTA in non-sensitized persons; and (3) to determine the sensitizing effect of LtSTA on the outcome of repeat tests at 30 and 60 days.		
Methodology: This trial will involve 36 healthy adult volunteers without previous exposure to *Leishmania* species, 12 in each of three cohorts. To qualify for enrollment, volunteers must meet the inclusion criteria, pass a medical examination, have acceptable vital signs, and have at least one positive skin test to a DTH antigen control to ensure that their cellular immune system is functioning properly. Volunteers who qualify for enrollment will be assigned to one of three cohorts with each cohort consisting of twelve subjects. Cohort 1 will receive the 15μg dose, cohort 2 will receive the 30μg dose and cohort 3 will receive the 50μg dose. As volunteers enter the study, they will be assigned to one of the three cohorts on a rotational basis. This procedure will be followed to maintain an equal number of volunteers in each cohort during the course of the study. The volunteers, principal investigator and study staff will be blinded as to the dose of LtSTA administered. A color code will be assigned to each concentration of LtSTA, as well as to the placebo and saline control for each cohort.		

Sponsor: Allermed Laboratories, Inc	BB IND 11822 LtSTA-08, Rev. 03 San Diego	
Name of Finished Product: *Leishmania tropica* Skin Test Antigen (LtSTA)		
Active Ingredient: *Leishmania tropica* Promastigote Lysate		

Number of Subjects: 36 healthy adult volunteers without previous exposure to *Leishmania* species, 12 in each of three cohorts.

Diagnosis and Main Criteria for Inclusion:

All volunteers will be subjected to a physical examination and laboratory work-up. The criteria for enrollment is the same in each group.

Inclusion Criteria: Male or Female in good health; Age 18 – 60 years; No past history of leishmaniasis or prior participation in a *Leishmania* study; No prior skin test with a *Leishmania* antigen; No occupational, residential, or travel exposure to *Leishmania*; Positive Candin® or Trichophyton skin test (≥ 5 mm induration).

Exclusion Criteria: History of adult atopic dermatitis, contact dermatitis to multiple agents, unexplained urticaria, or asthma; active allergic rhinitis or conjunctivitis; history of allergy or reactions to phenol, polysorbate 80, or glycerol; Medications: currently taking (within the last month) antihistamines or recent history of taking (within the last 1 year) corticosteroids, immunosuppressants; Splenectomy; Active medical disease*; Pregnancy or lactating; Immunization within 4 weeks; History of leishmaniasis; Occupational exposure to *Leishmania;* Prior participation in a *Leishmania* study; Prior skin test with *Leishmania* antigen; Travel history to *Leishmania* endemic areas; Abnormal screening lab results; Keloid scar formation

***Active Medical Disease**: Any active physical or psychiatric condition that may increase the risks associated with participation in the study or interferes with the interpretation of study results. Included chronic medical illnesses are cardiovascular disease, renal insufficiency, chronic respiratory illness, cirrhosis, chronic hepatitis, chronic pancreatitis, chronic diarrhea, malnutrition, malignancy, autoimmune disease, and asthma.

Test Product, Dose and Mode of Administration, Batch Number:

LtSTA is a clear, sterile solution containing the lysate of *L.tropica* promastigotes. The product is standardized by protein content, stabilized with buffered saline and preserved with 0.4% phenol. The dose is 0.1mL administered intradermally in the forearm. The product is provided to the clinical site at the concentration to be tested, *e.g.* 15µg/0.1mL (Cohort 1), 30µg/0.1mL (Cohort 2) and 50µg/0.1mL (Cohort 3).

Study Participant Duration of Treatment:

For each study participant the study will last approximately four (4) months. Participants will be skin tested on Visits 3, 6 and 9 of the study. The results of skin tests will be read after 48 hours (± 6 hours) on Visit 4, 7 and 10. A final evaluation is performed on Visit 11, 14 days after Visit 10 (see "Table: Time and Event schedule, page 28 of the Protocol).

Sponsor: Allermed Laboratories, Inc	BB IND 11822 LtSTA-08, Rev. 03 San Diego	
Name of Finished Product: *Leishmania tropica* Skin Test Antigen (LtSTA)		
Active Ingredient: *Leishmania tropica* Promastigote Lysate		

Reference Therapy, Dose and Mode of Administration, Batch Number:

See dose and administration above.

Criteria for Evaluation:

Efficacy: (1) The absence of induration ≥ 5mm to the first skin test with LtSTA demonstrating that a 15, 30 or 50μg dose does not elicit a false-positive DTH reaction; and (2) the absence of induration ≥ 5mm to a second and third skin test with LtSTA demonstrating that prior skin tests with a 15, 30 or 50μg dose of LtSTA do not cause sensitization.

Safety: The absence of local and/or systemic reactions to LtSTA including, but not limited to, tenderness or inflammation causing disability/incapacity lasting longer than 24 hours, necrosis at the skin test site, anaphylaxis, and death.

Statistical Methods:

Fisher's exact test will be used to calculate the 95% one-sided upper confidence limit (UCL) for sensitivity.

References:

1. STATA Statistical Software, Release 10, Stata Corp LP, College Station TX.
2. GW Snedecor & WG Cochran, Statistical Methods, 8th Edition, 1989, Iowa State University Press, Ames, Iowa
3. JL Fleiss, Statistical Methods for Rates and Proportions, 2nd Edition, 1981, John Wiley & Sons, New York, NY.

The table below shows the 95% one-sided upper confidence limit (UCL) when 10 subjects are skin tested and subjects give a positive result (R). This table shows R from 0 to 3. The data in the table are applicable to all three skin test doses. If R = 3 for a given dose at any time point in the study, this dose will be considered unacceptable for further development. Note: twelve (12) subjects will be enrolled in each cohort to allow for two (2) dropouts per cohort. If dropouts do not occur, the first ten (10) subjects enrolled in such cohort will be included in the statistical analysis of data.

Table. One-sided 95% upper limit for sensitizing rates

No. of Skin Tests (N)	Sensitized		One-sided Upper 95% (UCL) for Sensitizing Rate (%)
	R	Observed Rate (%)	
10	0	00.0	25.9
10	1	10.0	39.4
10	2	20.0	50.7
10	3	30.0	60.7

Sponsor: Allermed Laboratories, Inc	BB IND 11822 LtSTA-08, Rev. 03 San Diego	
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Active Ingredient: *Leishmania tropica* Promastigote Lysate		

Outcomes

The most favorable outcome of this investigation would be a negative skin test on the initial skin test and on both the first and second repeat skin tests with 50μg LtSTA, since this dose elicited positive skin tests in 100% of subjects with healed cutaneous leishmaniasis who failed to react to the 30μg dose of LtSTA. Although not ideal, this dose might be considered acceptable for further development if the first repeat skin test was negative followed by a positive test on the second repeat test. The 30μg dose would be the dose of choice if the 50μg dose is sensitizing, and only if the number of negative repeat skin tests with 30μg is greater than the number of negative repeat tests with the 50μg dose. The 15μg dose would be considered for development if both the 30 and 50μg doses were sensitizing after the initial skin test. If all three doses are sensitizing after the initial skin test, the 50μg dose will be adopted as a single use skin test antigen.

SUMMARY – CONCLUSIONS

Efficacy Results

The efficacy of LtSTA as a skin test antigen depends upon the sensitivity and specificity of the product. This study has been designed to determine if a 15, 30, or 50μg dose shows non-specific reactivity due to components of the antigen solution and if the product has the ability to sensitize lymphocytes of *Leishmania* naïve persons when administered intradermally. The presence or absence of a local inflammatory response to the first skin test with each of three doses of LtSTA will provide insight on the specificity of the antigen in a naïve population. The local inflammatory response to LtSTA following the first and second repeat skin tests will indicate if the antigen is sensitizing after intradermal administration.

Safety Results:

The safety of LtSTA as a skin test antigen depends upon the type and degree of adverse events associated with its use. Delayed-type hypersensitivity reactions to skin test antigens can range from mild itching, swelling, redness and pain to more severe local reactions, such as necrosis, at the test site. Systemic reactions also can occur including urticaria, gastrointestinal disturbances and respiratory distress leading to anaphylaxis. Study subjects will be monitored for these types of events after the first, second and third skin test. In addition, any change in laboratory findings will be evaluated following the administration of the three dose regime of LtSTA.

Sponsor: Allermed Laboratories, Inc	BB IND 11822 LtSTA-08, Rev. 03 San Diego	
Name of Finished Product: *Leishmania tropica* Skin Test Antigen (LtSTA)		
Active Ingredient: *Leishmania tropica* Promastigote Lysate		

CONCLUSION:

The information obtained from this investigation concerning the intradermal administration of LtSTA at a low, medium and high dose is critical to the design of a phase III clinical trial and the final labeled use of the product. The objective of the study, as stated above, is to determine a dose of LtSTA that does not cause false-positive reactions in *Leishmania* naïve individuals and does not sensitize recipients after intradermal administration.

3. ABSTRACT

This phase II study will evaluate 15μg, 30 μg and 50μg doses of *Leishmania tropica* Skin Test Antigen (LtSTA) for the ability to elicit positive delayed-type hypersensitivity (DTH) skin test in persons who have not been exposed to the *Leishmania* parasite. Each dose will be administered intradermally at 0, 30 and 60 day time points. The test will be read 48 hrs after administration and scored as positive or negative based on the presence or absence of induration > 5mm at the test site. The study will be blinded and placebo controlled to avoid bias in the reading and interpretation of the test results. Qualified subjects will be enrolled in the study and assigned to one of the three dose cohorts according to a randomized schedule. Persons assigned to the 15μg cohort will receive a 15μg dose on days 0, 30 and 60. Persons in the 30μg and 50μg cohorts will receive intradermal injections of 30μg and 50μg, respectively, on days 0, 30, and 60.

This study is being conducted for the purpose of determining if the 15μg, 30μg and 50μg doses of LtSTA cause induration at the skin test site in naïve persons. Based on previous studies conducted by Allermed, it is not anticipated that naïve subjects will react to the first intradermal injection of each dose of LtSTA. However, it is unknown if a second or third skin test with the same dose will result in a positive inflammatory response, due to the potential sensitization of lymphocytes from the first skin test. In a phase I safety study conducted by Allermed, evidence was found that a 120μg dose of LtSTA might have sensitized two of eight persons who were skin tested with that dose. In these individuals, the 48 hour reading was negative, but an inflammatory response developed at the site of the skin test after 10 to 14 days. This observation suggested the possibility that lymphocytes had been sensitized during this 1 to 14 day period after the skin test and were interacting with residual antigen in the skin.

In 2007, a 30μg dose of LtSTA was tested in 40 persons living in the Sidi Bouzid Region of Tunisia with healed cutaneous leishmaniasis caused by *Leishmania major*. This dose elicited a positive DTH skin test response in 34 individuals (85%). The six persons who were skin test negative to the 30μg dose were retested with a 50μg dose of LtSTA approximately 3 months later and found to be skin test positive. This finding suggested that either the 30μg dose was too small to elicit a positive skin test in some individuals, or the 30μg dose was responsible for sensitizing the lymphocytes of these subjects which resulted in a positive test to the 50μg dose.

Before beginning a phase III trial involving a population of subjects that will provide acceptable statistical power for a sensitivity/specificity study for either a 30μg or 50μg dose of LtSTA, it is advisable to first test both doses for sensitizing properties. The indication of LtSTA as a skin test antigen for single or multiple use will depend upon the ability or inability of the antigen to sensitize the lymphocytes of naïve individuals. To determine if sensitization is dose dependent, a 15μg dose also will be evaluated following the three injection procedure at 0, 30

and 60 days. The outcome of this investigation will be used to design a phase III trial using the highest dose of LtSTA that does not elicit a positive DTH skin test in naïve subjects, but reacts in a significant percent of individuals who have been clinically exposed to *Leishmania.*

4. LIST OF ABBREVIATIONS AND DEFINITIONS

AE	Adverse event – any untoward medical occurrence in a clinical study subject administered a pharmaceutical product and that does not necessarily have a causal relationship with this treatment.
BCA	Bicinchoninic acid assay – test for protein
CL	Cutaneous leishmaniasis
CRF	Case Report Form
DTH	Delayed-type hypersensitivity
Essential documents	Documents that individually and collectively permit evaluation of the conduct of a study and the quality of the data produced (see section 8 of ICH E6 Guideline for Good Clinical Practice).
GCP	Good Clinical Practices as outlined in ICH E6 Guidance Document
cGMP	Current Good Manufacturing Practice - 21Code of Federal Regulations Part 211
HRPO	Human Research Protections Office
Informed Consent	A process by which a subject voluntarily confirms his/her willingness to participate in a clinical trial, after having been informed of all aspects of the trial. Informed consent is documented by means of a written, signed, and dated informed consent form.
IRB	Institutional Review Board
LtSTA	*Leishmania tropica* Skin Test Antigen
Non-reacting dose	A dose that produces an area of edema < 5mm in diameter
Placebo	Buffered saline solution containing the same ingredients as LtSTA except for the *L.tropica* lysate.
SAE	Serious adverse event
USAMRMC HURO	U. S. Army Medical Research and Materiel Command Human Use Review Office
USAMRMC ORP HRPO	U. S. Army Medical Research and Materiel Command Office of Research Protections, Human Research Protections Office

5. BACKGROUND

5.1 MILITARY RELEVANCE

Leishmaniasis is a threat to soldiers deployed to endemic areas where the disease can cause significant morbidity in immunologically naïve individuals. Reports from Iraq have raised concerns regarding the potential impact of cutaneous leishmaniasis on deployed personnel and disruption to unit readiness. Past experience with this parasite has highlighted several areas of concern that a tuberculin-like skin test antigen of *Leishmania* could address. The antigen is easy to administer and may be used both diagnostically to confirm CL in military personnel with skin lesions and as a screening tool for asymptomatic undiagnosed cases in personnel returning from endemic areas. Improved methods also are needed to protect the health and blood supply of active and returning military personnel serving in *Leishmania* endemic regions of the world that are important to the national interests of the United States. Several *Leishmania* skin test antigens developed by the U.S. Army have successfully undergone phase I clinical testing. In 2000, Allermed was contracted by the U.S. Army to develop an FDA approved skin test antigen for military use. The *Leishmania tropica* Skin Test Antigen manufactured by Allermed, has been tested in humans in a phase I safety trial and in a phase II trial in which the sensitivity and specificity of the antigen were evaluated in persons with cutaneous leishmaniasis (CL) caused by *L.major*. Positive skin tests (induration ≥ 5mm after 48 hours) were observed in 100% of persons with active CL and in 85% of persons with a history of healed CL caused by *L.major* who were skin tested with a 30μg dose of LtSTA. The DTH reactivity of LtSTA manufactured from promastigotes of *L.tropica* in *L.major* infected persons indicates that the skin test antigen is cross-reactive with *L.major* and has potential utility in detecting exposure to other *Leishmania* species.

5.2 OVERVIEW

5.2.1 Investigational Product

Leishmania tropica Skin Test Antigen (LtSTA), is a sterile injectable microfluidized lysate of *Leishmania tropica* (WR#1063:C1A) promastigotes. The product is heat-treated, filtered, and formulated to a protein concentration of 15μg, 30μg and 50μg/0.1mL with 0.85% sodium chloride, 0.4% phenol, 0.01% Tween-80®, and 1% glycerin in phosphate buffer. The antigen is manufactured in compliance with current Good Manufacturing Practices (cGMP) at Allermed Laboratories, San Diego, CA 92111 (see section 10.5 for descriptions of product, placebo, saline and DTH controls).

5.2.2 Purpose of Clinical Trial

The purpose of the trial is three-fold as follows: (1) to evaluate the safety of 15μg, 30μg and 50μg/0.1mL doses of LtSTA in healthy adult volunteers with no known previous exposure to *Leishmania* parasites; (2) to provide information on the occurrence of false-positive skin tests to the initial dose of LtSTA in *Leishmania* naive persons; and

(3) determine the effect of previous skin tests with each dose of LtSTA on the outcome of repeat tests at 30 and 60 days.

5.2.3 Desired Outcome

Identify the highest dose of LtSTA that is safe and does not elicit false-positive DTH reactions and is non-sensitizing following its use as a skin test antigen.

6. INTRODUCTION

Leishmaniasis is a common parasitic disease occurring throughout Africa, Asia, and Latin America. [1-4] The life cycle of the *Leishmania* parasite is complex. The promastigote (form of the parasite found in the insect vector) is normally transmitted to humans by the bite of a female sand fly. Once introduced into a human, promastigotes attach themselves to and invade cells of the mononuclear phagocytic system. After entering macrophages, promastigotes change into amastigotes (the intracellular form of the parasite) and propagate inside a parasitophorous vacuole. The infected macrophage eventually bursts, releasing the amastigotes, which can then infect other target cells. After a blood meal from an infected host, amastigotes are released into the gut of sand flies where they mature into infective promastigotes ready to repeat the cycle.

Infection with *Leishmania* can result in a variety of clinical syndromes, conventionally divided into four major clinical groups: cutaneous, mucocutaneous, diffuse cutaneous, and visceral. The spectrum of disease is so wide that the result of an untreated infection with *Leishmania* ranges from asymptomatic to fatal disease. [5-7] Immunity is largely cell mediated; therefore, tests designed to detect cell-mediated immune responses in exposed individuals are more likely to represent true measures of infection. The use of a skin test to detect delayed-type hypersensitivity (DTH) is a convenient, simple and cost-effective method to assess cell-mediated immune response in humans and can be used for large-scale population surveys. [8] Although there are other methods of detecting cell-mediated immunity, these are difficult to perform, difficult to standardize, and are not practical for screening large numbers of individuals.

Many different skin test preparations have been used in endemic areas in past decades. Most investigators use a locally acquired strain of *Leishmania* to make a crude antigen preparation of whole promastigotes, some form of disrupted promastigotes, or a soluble promastigote antigen. These preparations lack standardization, have unknown sensitivity and specificity, unknown sensitizing capacity, and an undefined dose-response profile between the antigen content and the clinical syndrome or parasite load. In addition, no preparation has been made under a GMP regulatory environment that would allow use in the United States as an investigational new drug or as a commercially available product. Currently, there are several *Leishmania* skin test antigens in use worldwide but none is approved for use in the USA.

7. RISK ASSESSMENT AND PRECAUTIONS

Table: Risk Assessment

Procedure	Risks	Measures to Minimize Risks
Voluntary participation in investigational research project	Breach in confidentiality Anxiety	All laboratory specimens, evaluation forms, reports, and other records will be identified in a manner designed to maintain patient confidentiality. Records will be stored in a secure location with restricted access.
Administration of skin test antigens	Potential risks include: Swelling, painful arm(s), redness at the injection site, pruritis (urge to scratch), difficulty breathing, faintness, flushing, dizziness, weakness, tachycardia, abdominal cramps, itching, induration, nausea, flu-like symptoms, redness, blistering, necrosis, hives, headache, anaphylaxis, vesiculation, tenderness, regional adenopathy or lymphadenopathy (swelling of the lymph nodes), rash, local tissue necrosis, scar formation	Stopping rules are clearly defined. Medical Monitor oversight. Administration of test products by personnel who are properly trained to perform the function. Study subjects will be required to wait in the physician's office for 60 minutes post-administration of the test product. Emergency equipment and personnel will be immediately available at the study site.
Blood Draws	Bruising and bleeding around the site, discomfort, fainting and, rarely, infection	Blood to be drawn by a person trained in phlebotomy using aseptic technique.
Study participation includes HIV, pregnancy and hepatitis testing	Breach in confidentiality Psychological strain if there is a breach in confidentiality. A breach regarding a subject's HIV, pregnancy and/or hepatitis status could have a major impact on both a personal and professional level.	Names and other identifiers will not be directly used on data and specimens to ensure confidentiality of participants. Only investigators will be able to cross match information with participant identity for the purposes of clinical care.

Use of a crude *Leishmania* antigen to elicit DTH in infected individuals was first reported by Montenegro in 1926. [9] Since then many different *Leishmania* skin tests have been given to humans around the world without any reported significant systemic or local reaction. Thousands of doses of various *Leishmania* skin test antigens have been administered for epidemiological surveys and to test seroconversion after administration of vaccines and drugs for leishmaniasis. [10, 11, 12, 13]

The product that will be used in this clinical trial is produced in compliance with cGMPs and will meet requirements for safety, purity, identity and potency prior to use.

Study participants will be instructed to seek medical care at the closest emergency room if a systemic or serious local adverse reaction to the skin test occurs during the time periods between office visits. Participants will be asked to notify the study physician of any serious adverse event as soon as possible.

8. BENEFITS TO SUBJECTS AND COMMUNITY

The study sponsor will compensate subjects for the time lost during the different visits as well as the cost of transportation to the study site. A free copy of the results of the biological tests will be supplied to the subject if requested for his/her medical records. Information regarding any concomitant or intercurrent illnesses detected during the screening will be made available to study subjects. Potential benefits to the community include the availability of an FDA licensed skin test that can be used in the detection of exposure to *Leishmania* parasites.

9. JUSTIFICATION FOR THE ROUTE OF ADMINISTRATION AND DOSAGE

Skin test antigens by nature are given intradermally so that induration can be evaluated. The volume of the injection is 0.1mL. The doses to be administered in this study were chosen based on: (1) the results of previously published studies by others in which antigenic preparations similar to LtSTA were tested in humans; and (2) the results of phase I and II clinical trials conducted by Allermed.

9.1 PREVIOUS STUDIES BY OTHERS

To estimate a dose range for the clinical trials conducted by Allermed, published dose-response studies performed with *Leishmania* skin test antigens on humans with active or healed *Leishmania* infections were reviewed. In a dose-response study published by Reed et al., three of five doses administered were based on protein content (dosage of the other two antigens was based on parasite concentration). [14] The doses were 5μg, 25μg, and 50μg of protein, assayed by the bicinchoninic acid assay (BCA). [15] The resulting mean indurations for these doses were 7, 14, and 17mm, respectively. No adverse events were reported by these investigators.

Using these data, a dose-response line was constructed by plotting dose against mean induration. Linear regression analysis yielded a line with the equation $y = 0.2226x + 6.796$ (R^2=0.9258). By solving for the equation using the target average response high and low values, it was determined that induration of 15mm corresponded to a dose of 36μg and induration of 18mm corresponded to a dose of 50μg.

Two *Leishmania* skin test antigens similar to Allermed's LtSTA also were taken into account. These were products developed by the U.S. Army: one for *Leishmania tropica* and one

for *Leishmania mexicana*. Both of these products were demonstrated as safe in phase I clinical trials when administered at a concentration of 35μg of BCA protein.[16, 17]

9.2 PREVIOUS STUDIES BY ALLERMED

Prior to choosing doses of LtSTA for a phase I safety study, Allermed established criteria to define an appropriate dose range for both the phase I and phase II study designs. These criteria included: (1) a four-concentration dose-response curve, (2) the highest product dose targeted near the Ninhydrin equivalent for an 18mm induration, (3) the highest product dose targeted for safety purposes, and (4) a 2-fold difference between the four concentrations. Taking these criteria into consideration, doses of 20μg, 40μg, 80μg, and 120μg were chosen for the phase I study. The 120μg dose fell outside the 2-fold dilution scheme, but was selected in preference to a 160μg dose for safety purposes. Using the dose response curve from Reed et al.,[14] the two highest doses chosen by Allermed were predicted to fall within the targeted 15 – 18mm response range (Table 1). Additionally, the highest dose chosen by Allermed (estimated to be 46μg of BCA protein) was lower than the highest dose used by Reed, et al. (50μg of BCA protein).

Table 1. Equivalent BCA dose and calculated mean induration for clinical doses used by Allermed. For each clinical trial dose chosen, the equivalent BCA dose was calculated. The expected induration was then calculated using the dose-response line from Reed, et al.[14]

Ninhydrin Dose	Equivalent BCA Dose	Predicted Mean 48-hour Induration
20μg	22μg	11mm
40μg	27μg	13mm
80μg	37μg	15mm
120μg	46μg	17mm

The phase I safety trial conducted by Allermed in 2005 involved 32 healthy adult volunteers without known previous exposure to *Leishmania* parasites. Four doses of LtSTA (20μg, 40μg, 80μg and 120μg) were evaluated for safety in terms of adverse local and systemic events associated with the intradermal injection of 0.1mL of the antigen. No serious adverse events were observed in this trial. However, in two of eight volunteers the 120μg dose caused an induration response after two weeks which resembled a positive DTH reaction. This observation was believed to be the result of retained antigen at the skin test site to which the individuals developed sensitivity. Based on these findings, Allermed elected to use doses of 10μg, 20μg, 40μg and 60μg in a phase II study conducted in 2007.

The 2007 phase II trial involved 100 volunteers from the Sidi Bouzid Region of Tunisia. This area is highly endemic for *Leishmania major*, resulting in numerous cases of cutaneous leishmaniasis (CL) each year. According to local health authorities, leishmaniasis is second only to tuberculosis as a public health problem. From this population, twenty (20) adult volunteers

with active CL were tested with doses between 10μg and 80μg to obtain data that could be used in developing a dose-response line for LtSTA. These data are shown below in Table 2.

Table 2. LtSTA Doses and individual induration responses

Dose (μg/0.1mL)	Volunteer	Induration (mm)	Dose (μg/0.1mL)	Volunteer	Induration (mm)
10	1	6.5	40	11	17.5
	2	10.0		12	17.5
	3	14.0		13	25.0
	4	16.0		14	21.0
	5	5.5		15	15.0
20	6	20.0	80	16	25.5
	7	16.0		17	21.5
	8	9.5		18	21.0
	9	10.0		19	32.0
	10	16.0			

Table 3 shows the mean induration by dose.

Table 3. Mean induration by dose

Dose (μg/0.1mL)	Log (Dose)	Mean Induration (mm)
10	1.0	10.4
20	1.3	14.3
40	1.6	19.2
80	1.9	25.0

Doses were then converted to logarithm to the base 10. The best fit linear regression Y on X was calculated, where X is log (dose) and Y is the average induration at that dose. The results were:

Slope B = 16.2 Y-intercept A = -6.3 and R-squared = 0.99

The slope was highly significantly different from zero with p-value = 0.004. R-squared was 0.99. Using this linear regression line, Y = - 6.3 + 16.2 X, the log (dose) corresponding to various induration sizes from 15mm to 18mm was calculated. These data are shown in Table 4.

Table 4. Estimated doses

Induration (mm)	Log (dose)	Dose (μg/0.1mL)
15	1.314	21
16	1.376	24
17	1.438	27
18	1.499	32

The four doses of LtSTA that were used to develop a dose-response line in Allermed's phase II trial were selected on the basis of calculations made from data reported by Reed *et al.* [14] These doses were tested for safety in Allermed's phase I trial and, based on the outcome of

the phase I trial, the range was modified downward from 20μg -120μg to 10μg -80μg. As shown in Table 1, the estimated range of induration expected from the data reported by Reed *et al.* was 11-17mm. The mean induration response for each of the corresponding Allermed doses was higher, but the range of reactivity was similar in both studies. Induration was not observed to either the saline control or to placebo in the cohort of twenty (20) subjects with active CL who participated in the titration of LtSTA in the phase II trial.

Although the targeted induration response was 15mm with a corresponding dose of 21μg/0.1mL, a dose of 30μg/0.1mL (approximately 18mm) was selected for further study. Part of the reasoning behind this decision was based on the observation that the 20μg/0.1mL (Table 2) elicited induration in two subjects of 9.5mm and 10mm which were deemed to be borderline small. The sensitivity and specificity of the 30μg/0.1mL dose was evaluated in forty (40) volunteers with a history of active CL within the past two years (Cohort 1) and forty (40) volunteers without known previous exposure to the parasite (Cohort 2). Within Cohort 1, 85% (34/40) reacted with a positive DTH response to 30μg LtSTA. Within Cohort 2, 97% (39/40) gave a negative response to the 30μg dose. Questioning of the single volunteer with a positive skin test to LtSTA in the latter group revealed that exposure to the parasite was probable based on the individual's history.

To obtain additional information regarding the six volunteers in Cohort 1 with negative skin tests to the 30μg dose of LtSTA, a follow up investigation was conducted in which these individuals were skin tested with 50μg LtSTA. Blood samples were also obtained for lymphocyte proliferation studies using LtSTA and an antigenic preparation of *L.major*. The results of this investigation revealed the following: 1) all six individuals had a positive DTH skin test response to the 50μg dose, and 2) all six individuals had lymphocyte proliferation to LtSTA and to *L.major* antigen. However, the degree of proliferation to *L.major* antigen was noticeably higher than proliferation to LtSTA, indicating a higher degree of sensitivity to the homologous antigen system. Until the extent of cross-reactivity between LtSTA and other species of *Leishmania* is known, the potential for false-negative outcomes must be considered before antigen is used as a means of detecting infection or prior exposure to species other than *L.tropica* and *L.major*.

At least two possibilities exist to explain why positive skin tests to a 50μg/0.1mL dose of LtSTA were observed in persons who were skin test negative to a dose of 30μg/0.1mL: (1) The sensitivity to antigens in LtSTA was too low to be detected with a 30μg/0.1mL dose, and (2) positive skin tests to the 50μg/0.1mL dose were the result of priming (sensitization) of the immune system with the initial 30μg/0.1mL dose. Jose *et al.* [18] found that 33% of subjects who were skin test negative to an undefined crude *Leishmania* antigen made from *L.amazonensis* became skin test positive upon retest to the same antigen after 30 days and 67% were positive after 90 days.

Considering the proposed use of LtSTA by the U. S. military, *i.e.* to test personnel prior to and after deployment to *Leishmania* endemic regions of the world, it is imperative to know if conversion from a negative to a positive skin test occurs after an initial skin test with LtSTA has been administered. The proposed study has been designed to evaluate three doses of LtSTA for safety, false-positive responses and sensitizing properties. The basis for electing three doses is explained below.

15μg/0.1mL dose: From the dose-response data reported in Table 3, the induration response to 15μg is estimated to be between 10mm and 14mm. This dose contains less *Leishmania* protein and, therefore, should be less immunogenic than a 30 or 50μg dose, thereby reducing the possibility of sensitization from a previous skin test.

30μg/0.1mL dose: This dose was evaluated for sensitivity (positive skin test in persons with healed CL) and specificity (negative skin test in persons with no prior known exposure to *L.major*) in the phase II trial. The observed sensitivity was 85% in subjects with a history of cutaneous leishmaniasis (CL) and the observed specificity was 97% in persons from an endemic area without a history of CL. It is essential to evaluate a 30μg dose for sensitizing properties before a phase III trial is designed.

50μg/0.1mL dose: This dose elicited positive skin tests in phase II subjects with a history of cutaneous leishmaniasis who were skin test negative to the 30μg dose. From the phase II results, the 50μg dose hypothetically could detect past exposure to *Leishmania* in 100% of subjects. Therefore, as with the 30μg dose, additional information is needed on the sensitizing properties of 50μg LtSTA before a phase III trial is designed.

10. STUDY DESIGN

10.1 GCP STATEMENT

The principal investigator has reviewed this protocol and will conduct the study in full compliance with current Good Clinical Practice Guidelines and FDA regulations as indicated by his signature on the protocol signature page.

10.2 POPULATION TO BE STUDIED

A total of 30 volunteers and six (6) alternates ages 18-60 years, inclusive of gender, race and socioeconomic status, will be enrolled in the study following their expressed consent. They will be recruited from the local community by non-coercive means. Refer to Section 11.1 of the protocol for the recruitment plan. Data analysis will be performed on the first thirty (30) persons, ten (10) per cohort, that complete the four month trial. The six alternate subjects will be enrolled to provide a final count of thirty (30) in the event that some subjects drop out.

Each volunteer will be assigned a number upon screening. The numbering system consists of an alpha/numeric combination (Lt08) followed by a two-digit number (01) as a suffix. As an example, subject number 1 will be assigned subject number Lt08-01. The number that is assigned to a subject will be used for that subject throughout the study.

Subjects will be screened for residential, occupational and travel history to exclude persons that have resided, worked, or traveled in geographic areas that are endemic for *Leishmania* parasites. To qualify for enrollment, volunteers must meet the inclusion and exclusion criteria (see section 11.4 and 11.5), pass a medical examination, have acceptable vital signs, and have at least one positive skin test to a DTH antigen control to ensure that their cellular immune system is functioning properly.

Volunteers who qualify for enrollment will be assigned to one of three cohorts with each cohort consisting of ten subjects. Cohort 1 will receive the 15μg dose, cohort 2 will receive the 30μg dose and cohort 3 will receive the 50μg dose. As volunteers enter the study, they will be assigned to one of the three cohorts on a rotational basis. This procedure will be followed to maintain an equal number of volunteers in each cohort during the course of the study. The volunteers, principal investigator and study staff will be blinded as to the dose of LtSTA administered. A random code will be assigned to each concentration of LtSTA, as well as to the placebo and saline controls for each cohort (see sec. 10.5.5 "Blinding of Investigational Products").

The minimum legal age for adult enrollment is 18 years. Persons over 60 years of age may have reduced cellular immunity which could potentially affect study results.

10.3 OBJECTIVES

The primary objective of this study is to identify a dose of LtSTA that can be used in a phase III trial involving both infected and non-infected subjects. To qualify, the dose must be: (1) safe, (2) free of non-specific reactive substances with the potential to cause false-positive reactions, and (3) non-sensitizing, *i.e.*, does not convert negative skin test responders to positive skin test responders. To accomplish this objective, three doses of LtSTA will be evaluated for safety, false-positive tests and sensitizing properties.

10.4 PRECAUTIONS AND ENDPONTS

10.4.1 Precautions

1. Subjects should not receive any vaccine 4 weeks prior to the start of the study, during the study, and for at least 2 weeks after LtSTA is administered without discussing it with the principal investigator.

2. Medications (including over-the-counter medicines, such as aspirin, acetaminophen [Panadol, Tylenol] or ibuprofen [Brufen]) should not be taken within two days of

starting the study or during the study without discussing the use of these medications with the principal investigator.

10.4.2 Study Endpoints

Safety: The absence of local and/or systemic reactions to LtSTA including, but not limited to, tenderness or inflammation causing disability/incapacity lasting longer than 24 hours, necrosis at the skin test site, anaphylaxis, and death. If the safety endpoints fail, the study will be stopped.

Efficacy: (1) The absence of induration ≥ 5mm to the first skin test with LtSTA demonstrating that a 15μg, 30μg or 50μg dose does not elicit a false-positive DTH reaction; and (2) the absence of induration ≥ 5mm to a second and third skin test demonstrating that prior skin tests with a 15μg, 30μg or 50μg dose of LtSTA does not cause sensitization. The testing of each dose will proceed until ten subjects receiving the dose have completed the protocol. A "non-reacting dose" is one that produces an area of induration < 5mm. Testing of individual subjects will be stopped if the subject has a positive DTH response to the initial or first repeat skin test.

10.5 INVESTIGATIONAL PRODUCTS AND CONTROLS

10.5.1 Description of LtSTA

LtSTA will be manufactured by Allermed according to approved procedures and under cGMP and supplied at a target protein concentration of 15, 30 and 50μg/0.1mL in 2cc vials each containing 1mL of product.

10.5.2 Description of Placebo

The placebo control will be manufactured by Allermed according to approved procedures and under cGMP. The *Leishmania tropica* Skin Test Placebo is formulated to contain all the components of LtSTA except the parasite lysate. The placebo consists of 0.85% sodium chloride, 0.4% phenol, 0.01% Tween-80®, and 1% glycerin in a 25mM phosphate buffer.

10.5.3 Description of the Saline Control

The saline control containing 0.9% sodium chloride (and 0.4% phenol as the preservative) will be manufactured at Allermed.

10.5.4 Description of the Anergy Panel (DTH Controls)

Candin® and Trichophyton allergenic extract (1:500 w/v) will be used as DTH controls for testing the cellular hypersensitivity of study volunteers. Candin® is an FDA approved product manufactured by Allermed Laboratories, Inc. that can be used to detect delayed-type hypersensitivity to the yeast *Candida albicans*. Trichophyton is an FDA approved allergenic extract with DTH properties, manufactured by Allermed

Laboratories, Inc. This extract is manufactured from the fungi *Trichophyton rubrum* and *Trichophyton mentagrophytes*, causative agents of dermatophytosis of the hair, nails, and skin. It is expected that between 70% and 80% of trial volunteers with functional cellular immunity will produce a positive DTH response to one or both of the positive control antigens, demonstrating that these individuals are capable of mounting an appropriate cellular immunological response to a DTH antigen. If a volunteer demonstrates a positive response to one of the controls (either Candin® or Trichophyton) then that control shall be used as a positive control for that individual for the remainder of the study. If the volunteer demonstrates a positive response to both controls, Candin® shall be used as the positive control for that individual for the remainder of the study.

10.5.5 Blinding of Investigational Products

LtSTA, placebo and saline vials will be coded specific to each cohort. The true identity of each vial will be recorded and enclosed in a sealed envelope and given to the principal investigator with instructions to open the envelope only if an emergency occurs that requires identification of the investigational products. The positive control (Candin® or Trichophyton) will not be blinded and will be administered as a single injection in the opposite forearm from the three investigational products.

10.5.6 Investigational Product Accountability

All study products and drugs for anaphylaxis will be kept in a secure location at the clinical site. IND materials will be logged into the clinic on arrival from Allermed. A Chain of Custody Form will accompany the investigational products and will become part of the study file. A written log will be maintained and only an authorized person will remove the products from storage for use in the study. The site must contain a monitored refrigerator unit capable of storing the test articles at 2 - 8°C before and after use. A temperature recording devise will be supplied by Allermed. This devise will remain in the storage refrigerator with the product and controls for the length of the study. When the study is complete, the temperature recording devise shall be returned to Allermed.

10.6 PROCEDURES

10.6.1 Testing to be Performed on Blood and Urine

A comprehensive metabolic panel (Table 5), CBC with differential, urinalysis, HIV, Hepatitis B, and Hepatitis C assays will be performed. Pregnancy testing will be performed in the clinic using commercially available pregnancy test strips.

Table 5. Laboratory tests included in the comprehensive metabolic panel.

Albumin	Alkaline Phosphatase	Bilirubin-Total	Potassium
BUN	Calcium	CO2	Chloride
Creatinine	Glucose	GDT (AST)	
GPT (ALT)	Protein-Total	Globulin	
A/G	Bun/Crt	Sodium	

Blood and urine samples will be collected during screening (two 10mL tiger-top tubes for serum, one 5mL lavender-top tube for blood, and one 20mL yellow top container for urine). Approximately 10mL of blood and 20mL of urine will be collected during screening and at the end of study physical examination (one 5mL tiger-top tube for serum, one 5mL lavender-top tube for blood, and one 20mL yellow top container for urine).

Samples will be identified with the subject's ID and placed in plastic bags provided by Sharp Memorial Hospital Laboratory. A requisition form is enclosed in the bag with the sample. A courier employed by Sharp Memorial Hospital Laboratory collects the sample and transports them in a company vehicle to the laboratory. The samples will then be tested. Once the samples are tested and the report is issued, the samples will be destroyed and will not be used for further research.

All female study participants must either show medical documentation of surgical sterilization or take a urine pregnancy test within the 24 hours before skin testing.

10.6.2 Skin Tests

One-tenth milliliter of each test article will be injected intradermally into the alcohol-cleansed volar surface of the forearm under the supervision of a physician who will have drugs and equipment to treat anaphylaxis immediately available. Skin tests will be placed approximately 2 inches (5cm) apart to avoid overlapping reactions. The diameter of induration and/or erythema will be measured in millimeters at 30 minutes, (to detect immediate IgE hypersensitivity reactions), and at 48 ± 6 hours (induration consistent with DTH) by a combination of the "Sokal" method[30] and the FDA method (Docket No. 94N-0012). Induration and erythema will be measured at 48 ± 6 hours. The indurated border will be outlined as a solid line with a ballpoint pen. Erythema will be outlined with a broken (dotted) line. The tracing will be transferred to paper using transparent tape. The largest diameter and its orthogonal diameter of induration will be measured, and averaged. Erythema will be measured for informational purposes only. The skin test will be placed by a clinical researcher according to the position of the test article shown in the CRF for each subject. Care will be exercised to inject the same 0.1mL volume of the saline control, placebo and LtSTA, so that the 48 hour response to each reagent can be compared.

10.6.2.1 Performing the Test

The placement of the skin tests on the volar surface of the forearm will be predetermined for each study participant. The test is performed by inserting the needlepoint, bevel side up, into the skin at a 15-20 degree angle. The angular distance of penetration should be approximately 1.5mm to pass through the epidermis. This distance can be judged by inserting the needle into the skin until the bevel is no longer visible. Once the needlepoint is in the proper location, 0.1mL of antigen injected into the dermis, maintaining steady pressure on the plunger and slowly advancing the needle tip until the complete volume is injected and the needle is withdrawn. If the test is done correctly, a distinct, sharply defined bleb (5-10mm in diameter) will occur at the injection site. A tiny drop of test antigen at the injection site is not uncommon. The patient may feel a slight burning sensation.

10.6.2.2 Reading the Test

The DTH control skin tests shall be read at 48 ± 6 hours post injection. LtSTA, placebo, and saline control skin tests shall be read after 30 minutes for a wheal/flare response and after 48 ± 6 hours post injection for induration and erythema. The extent of induration is determined by palpating the skin test site with the index finger for firmness. The size of the area of firmness is independent of the erythematous response that may be associated with the reaction. Commonly, DTH reactions have a dense erythematous core surrounded by an area of firmness. The true induration response must include the entire area of firmness, regardless of erythema.

10.6.2.3 Recording the Test

Wheal/flare reactions after 30 minutes shall be reported as mm edema/mm erythema. Skin tests read at 48 ± 6 hours shall be recorded in mm induration using the ballpoint pen method. [19]

10.6.2.4 Measuring and Recording Induration

The area of induration (firmness) shall be outlined with a ballpoint pen. A permanent record of the reaction size shall be made by pressing transparent tape over the tracing and placing the tape on the skin test record. To determine the mean mm induration response, the tracing shall be measured at the greatest induration diameter and its orthogonal diameter and the mean of the two numbers should be calculated.

10.6.2.5 Measures taken to Minimize Bias

A randomization list will be prepared specifying which test article will be placed proximally, distally, or intermediate on the forearms.

10.6.2.6 Breaking the Randomization Code

In the event of an adverse event or unexpected circumstance that requires the identity of a study article (LtSTA or control) the list of coded articles shall be included in the Study Binders.

10.6.2.7 Identification of Source Data

All data will be recorded directly on Case Report Forms (CRFs) with the exception of the clinical laboratory results (computer printouts) which will be added to the CRF file for each volunteer. The volunteer's name on each laboratory result will be masked and replaced with the study number. Study numbers will be assigned at the pre-enrollment testing visit.

10.7 BREAKDOWN BY VISIT TO CLINICAL SITE

10.7.1 Visit #1

1. Complete consent forms, volunteer registration data form, volunteer reported medical history, and medical history review by physician.
2. Check and record vital signs and perform physical exam #1.
3. Perform urine pregnancy test #1 on non-surgically sterilized females.
4. Obtain blood and urine samples
5. Complete Eligibility Record for Study Enrollment
6. Administer Candin® and Trichophyton skin tests per protocol Section 10.6.2.1.
7. Check vital signs after skin testing
8. Complete post procedure volunteer evaluation Visit #1.
9. Give subject daily diary form to record AE until next appointment.

10.7.2 Visit #2 (48 ± 6 hrs after Visit #1)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Read and record skin test results per protocol Sections 10.6.2.2. and 10.6.2.3.
4. Check and record vital signs.
5. Complete post procedure volunteer evaluation Visit #2.
6. Note: Subject must have a positive skin test to Candin® or Trichophyton to continue in the study.

10.7.3 Visit #3 (14 ± 3 days after visit #2)

1. Review laboratory results on blood and urine samples. All values must be within accepted range for subject to continue in the study.
2. Check and record vital signs
3. Perform urine pregnancy test #2 on non-surgically sterilized females.

4. **Administer skin tests with drug products** (LtSTA, Placebo, and Saline) in the left forearm per protocol Section 10.6.2.1. Administer one positive control (either Candin® or Trichophyton) in the right forearm per Section 10.6.2.1.
5. Check and record vital signs 60 minutes post skin testing.
6. Complete post procedure volunteer evaluation Visit #3.
7. Give subject daily diary form to record AE until next appointment.

10.7.4 Visit #4 (48 ± 6 hrs after Visit #3)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Read and record skin test results per protocol Sections 10.6.2.2 and 10.6.2.3.
4. Check and record vital signs.
5. Complete post procedure volunteer evaluation Visit #4.
6. Give daily diary form to subject to record AE until next appointment.

10.7.5 Visit #5 (7 ± 3 days after Visit #4)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Check and record vital signs.
4. Complete volunteer evaluation Visit #5.

10.7.6 Visit #6 (30 ± 7 days after Visit #3)

1. Perform urine pregnancy test #3 on non-surgically sterilized females.
2. Check and record vital signs.
3. **Administer skin tests with drug products** (LtSTA, Placebo, and Saline) in the left forearm per protocol Section 10.6.2.1. Administer one positive control (either Candin® or Trichophyton) in the right forearm per Section 10.6.2.1.
4. Wait 60 minutes after skin testing before checking and recording vital signs.
5. Complete post procedure volunteer evaluation Visit #6.
6. Issue daily diary form to subject to record AE until next appointment.

10.7.7 Visit #7 (48 ± 6 hrs after Visit #6)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Read and record skin test results per protocol Sections 10.6.2.2 and 10.6.2.3.
4. Check and record vital signs.
5. Complete post procedure volunteer evaluation Visit #7.
6. Issue daily diary form to subject to record AE until next appointment

10.7.8 Visit #8 (7 ± 3 days after Visit #7)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Check and record vital signs.
4. Complete volunteer evaluation form.

10.7.9 Visit #9 (30 ± 7 days after Visit #6)

1. Perform urine pregnancy test #4 on non-surgically sterilized females.
2. Check and record vital signs.
3. **Administer skin tests with drug products** (LtSTA, Placebo, and Saline) in the left forearm per protocol Section 10.6.2.1. Administer one positive control (either Candin® or Trichophyton) in the right forearm per Section 10.6.2.1.
4. Wait 60 minutes after skin testing before checking and recording vital signs.
5. Complete post procedure volunteer evaluation Visit #9.
6. Issue daily diary form to subject to record AE until next appointment.

10.7.10 Visit #10 (48 ± 6 hrs after Visit #9)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Read and record skin test results per protocol Sections 10.6.2.2 and 10.6.2.3.
4. Check and record vital signs.
5. Obtain blood and urine samples.
6. Complete post procedure volunteer evaluation Visit #10.
7. Issue daily diary forms to subject to record AE until next appointment.

10.7.11 Visit #11 (14 ± 3 days after Visit #10)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Check and record vital signs.
4. Perform physical exam.
5. Perform urine pregnancy test #5 on non-surgically sterilized females.
6. Review lab results for blood and urine.
7. Complete end of study form. If follow-up is indicated, schedule subject for next appointment.

Table. Time and Event Schedule.

Visit Number	Time	Length of Visit	Events
1	0	2.5 hrs	Consent, history & physical exam, vital signs, blood draw, urine sample, urine pregnancy test (females), administration of DTH control skin tests for anergy. Issue daily diary forms.
2	48 ± 6 hrs after visit 1	30 min	Check vital signs. Read & record DTH control skin tests, review diary, complete study enrollment. Select appropriate DTH control for use during the remainder of the study
3	14 ± 3 days after visit 2	1.5 hrs	Urine pregnancy test (females), vital signs, **administration of LtSTA**, placebo, and saline control skin tests. Post injection vital signs after 60 minutes. Issue new daily diary. Post procedure evaluation.
4	48 ± 6 hrs after visit 3	30 min	Read & record LtSTA and control skin tests results, vital signs, review diary. Post procedure evaluation. Issue daily diary.
5	7 ± 3 days after visit 4	30 min	Review diary, vital signs. Complete volunteer evaluation form.
6	30 ± 7 days after visit 3	1.5 hrs	Urine pregnancy test (females), vital signs, **administration of LtSTA**, placebo, and saline control skin tests. Vital signs after 60 minutes. Issue new diary form
7	48 ± 6 hrs after visit 6	30 min	Read & record LtSTA and control skin tests results, vital signs, review diary, post procedure evaluation. Issue new diary form.
8	7 ± 3 days after visit 7	30 min	Review diary, vital signs. Complete volunteer evaluation form.
9	30 ± 7 days after visit 6	1.5 hrs	Urine pregnancy test (females), vital signs, **administration of LtSTA**, placebo, and saline control skin tests. Vital signs after 60 minutes. Issue new diary form.
10	48 ± 6 hrs after visit 9	30 min	Obtain blood & urine samples. Read & record LtSTA and control skin tests results, vital signs, review diary, post procedure evaluation. Issue new diary form.
11	14 ±3 days after visit 10	1.5 hrs	Review diary, vital signs and physical exam, complete adverse events form, complete volunteer evaluation, complete end of study form.

10.8 MONITORING OVERSIGHT

10.8.1 Subject Safety

Volunteers participating in the study will be monitored for vital signs that conform to study requirement at each visit. Signs and symptoms of adverse reactions during and after the performance of study procedures will be evaluated by study personnel. Daily diaries will be kept by volunteers to record adverse events that occur between scheduled visits. Blood and urine samples will be collected at the beginning and end of the study to determine if metabolic or pathological changes occur that could be related to the investigational products.

10.8.2 Investigational Site

Regular monitoring visits to the medical clinic conducting the protocol will be made by personnel assigned by Allermed to monitor the conduct and progress of the study. Interviews with the principal investigator and investigational staff will be held to evaluate the role of each person involved in the performance of study procedures and record keeping. Some monitoring visits will be made concurrently with visits by study volunteers to observe how the data required by the protocol is being obtained.

10.8.3 Source Documents

CRF's will serve as source documents unless information related to the study or well being of study volunteers can not be adequately captured on the forms provided. All documents relating to the study procedures and any documents that result from actions relating to the study will be archived by the principal investigator and, where appropriate, copies will be kept by Allermed in a secure location.

10.8.4 Analysis of Data

Data obtained from the study will be reviewed and analyzed by Allermed personnel or by consultants employed by Allermed. Personal information of study volunteers will be maintained on a confidential basis in compliance with the laws and regulations pertaining to patient privacy.

11. SELECTION, WITHDRAWAL AND REPLACEMENT OF SUBJECTS

11.1 RECRUITMENT PLAN

Volunteers will be recruited from the patient data base of the clinical site. If other recruiting measures are needed, such as placing an advertisement in the local newspaper or by posting or distributing flyers in public areas, such advertisement or flyers will be sent to the IRB for review and approval. Refer to Section 10.2 for specifics of the population to be studied.

11.2 INFORMED CONSENT PROCESS

Study volunteers will be consented by either the principal investigator or a clinical research associate. This will take place at the study site and volunteers will be given sufficient time to make an informed decision about participation in the clinical trial, including the ability to

take the consent forms home for review. Questions will be answered by either the principal investigator, clinical research associate, or an agent of the sponsor. Additionally, study recruits will be given the IRB contact information for questions relating to their rights as a research subject. As indicated in the consent form, information learned during the course of the trial which may affect a volunteer's decision to participate will be explained to all study volunteers.

Volunteers will be informed that the principal investigator may terminate their participation in the study without regard to their consent. The circumstances under which this may occur include a volunteer exhibiting behavior that may bias the study, demonstrating behavior indicating mental or emotional unfitness to continue the study, or nonconformance to the protocol requirements. In all cases both the principal investigator and medical monitor will make a joint decision as to whether the volunteer's participation in the study will be terminated.

11.3 EVALUATION BEFORE ENTRY

Each volunteer will sign an informed consent. Volunteers will undergo a medical history screening with special attention to a history of asthma, atopic skin disease and other allergic diseases. This will be followed by physical examination, and screening laboratory tests, including urine pregnancy test (if non-surgically sterilized female), and serological studies for hepatitis B, hepatitis C, and HIV infection. Female volunteers who are not surgically sterile will be asked to take a urine pregnancy test within 24 hours before anergy skin tests and LtSTA administration. All volunteers will sign an informed consent for HIV antibody testing (attached) and will undergo DTH (anergy) testing with Candin® and Trichophyton allergenic extract (1:500 w/v). Induration equal to or exceeding 5mm for at least one of the DTH control antigens will be evidence of normal delayed hypersensitivity.

11.4 INCLUSION CRITERIA

- Male or Female in good health
- Age 18 – 60 years
- No past history of leishmaniasis or prior participation in a *Leishmania* study
- No prior skin test with a *Leishmania* antigen
- No occupational, residential, or travel exposure to *Leishmania*
- Positive Candin® or Trichophyton skin test (≥ 5mm induration)

11.5 EXCLUSION CRITERIA

- History of adult atopic dermatitis, contact dermatitis to multiple agents, unexplained urticaria, or asthma
- Active allergic rhinitis or conjunctivitis
- History of allergy or reactions to phenol, polysorbate 80, or glycerol
- Medications: Currently taking (within the last month) antihistamines or recent history of taking (within the last 1 year) corticosteroids, immunosuppressants.
- Splenectomy
- Active medical disease *
- Pregnancy or lactating
- Immunization within 4 weeks
- History of leishmaniasis
- Occupational exposure to *Leishmania*
- Prior participation in a *Leishmania* study
- Prior skin test with *Leishmania* antigen
- Travel history to *Leishmania* endemic areas
- Abnormal screening lab results
- Keloid scar formation

***Active Medical Disease**: Any active physical or psychiatric condition that may increase the risks associated with participation in the study or interferes with the interpretation of study results. Included chronic medical illnesses are: cardiovascular disease, renal insufficiency, chronic respiratory illness, cirrhosis, chronic hepatitis, chronic pancreatitis, chronic diarrhea, malnutrition, malignancy, autoimmune disease, and asthma.

Pregnancy prevention: Females will be instructed to abstain from sexual relations or practice a method of birth control during the study and two weeks following the end of the study. They will further be instructed that except for surgical removal of the uterus, birth control methods such as the use of condoms, a diaphragm or a cervical cap, birth control pills, IUD, or sperm killing products are not totally effective in preventing pregnancy.

11.6 WITHDRAWAL CRITERIA

Volunteers will be allowed to withdraw from the study at any time without prejudice or loss of benefits to which they are entitled. Volunteers may be removed from the study by the principal investigator or the medical monitor should their continued participation be injurious to their health and well being at any time. The Human Research Protections Office (HRPO) and the local IRB will be notified whenever a subject is withdrawn from the study. Volunteers removed from the study subsequent to receiving the skin test will have a follow up visit after 48 hours ± 6 hours. Drop-out volunteers will be replaced. Although this study is of a relatively short duration, the dropout rate is expected to be very low.

11.7 REPLACEMENT CRITERIA

To ensure that 30 volunteers complete the study, six additional volunteers meeting all inclusion/exclusion criteria will be enrolled as needed to replace drop-outs.

11.7.1 Non-Qualifying Volunteers

Persons who sign the informed consent, but who do not qualify for enrollment in the study due to: (1) their failure to meet all inclusion/exclusion criteria or, (2) other documented conditions considered sufficient by the principal investigator or medical monitor to warrant the exclusion of the volunteer, will be informed that they cannot participate in the study. The reason for exclusion will be explained to the volunteer and appropriate recommendations will be provided by the principal investigator in consultation with the medical monitor. All records, laboratory reports, etc., relating to the enrollment process for the volunteer shall be kept and maintained with other study documents.

11.7.2 Volunteers with Concomitant or Intercurrent Illness Detected during Screening

Persons with concomitant or intercurrent illness that is detected during enrollment screening will be excluded from participation in the study. These individuals will be considered non-qualifying volunteers. The procedures that will be followed are the same as those described above for Non-Qualifying Volunteers.

12. MEDICATIONS

The drugs not permitted are described in the exclusion criteria Section 11.5. In addition, study volunteers will be encouraged to avoid all new prescription and over the counter medications during the 7 days after the LtSTA application, and will be asked to contact a study physician immediately should any new medications be prescribed.

13. ASSESSMENT OF SAFETY

Safety of the skin test is demonstrated by the absence of adverse events following skin tests with the investigational products. Adverse reactions will be classified as immediate, generalized/systemic, or local, and the reactions will be graded on a scale including mild, moderate, and severe. Occurrence of adverse events will lead to a review of the study for safety before testing any further subjects per the "Stopping Rules" in Section 13.6.

An adverse event temporally related to participation in the study should be documented whether or not considered to be related to the test article. This definition includes intercurrent illnesses and injuries, and exacerbation of preexisting conditions.

13.1 SERIOUS ADVERSE EVENTS (SAE)

A serious adverse event is any untoward medical occurrence that:

- Results in death
- Is life-threatening [A life threatening event is an event which presents the risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death had it been more severe.]
- Requires in-patient hospitalization
- Results in persistent or significant disability/incapacity [A disabling/ incapacitating adverse event is any event which may result in a substantial disruption of the volunteer's ability to carry out normal life functions. This definition is not intended to include minor cases of headache, nausea, vomiting, diarrhea, influenza, rhinorrhea, lacrimation or accidental trauma, such as a sprained ankle.]
- Results in a congenital anomaly
- Is serious based on the medical judgment of the principal investigator and/or the medical monitor or consulting physician

13.2 ADVERSE EVENTS REPORTED BY INVESTIGATOR OR PHYSICIAN

13.2.1 Immediate Reactions

The following reactions occurring within 60 minutes after injection of the investigational product will be considered mild, moderate, or severe as judged by a physician:

- Subjective feeling of intense anxiety or panic
- Flushing and sweating
- Onset of nausea, vomiting, cramps or diarrhea
- Onset of pruritus in skin or mucous membranes
- Onset of urticaria or angioedema
- Acute onset of rhinorrhea, coughing, wheezing, or dyspnea
- Progressive signs of an anaphylactic reaction
- Hypertension or hypotension

13.2.2 Generalized/Systemic reactions

The following reactions occurring between 30 minutes and 24 hours following injection of the investigational product will be considered mild, moderate, or severe as judged by a physician:

- Fever (temperature greater than 100° F) with or without chills
- Nausea, vomiting, abdominal cramps, diarrhea (acute onset of more than 2 watery stools within a 24 hour period)
- Wheezing or dyspnea
- Uticaria or angioedema

13.2.3 Local reactions

The following reactions occurring within two weeks following injection of the investigational product will be considered mild, moderate, or severe as judged by a physician.

Reactions generally considered mild

- Itching, burning, mild discomfort
- Erythema/edema < 30mm

Reactions generally considered moderate

- Noticeable discomfort, but not compromising limb function
- Erythema/edema ≥ 30mm and < 80mm
- Blistering at test site

Reactions considered severe

- Pain causing decreased motion and use of the limb
- Erythema/edema > 80mm
- Necrosis at the test site
- Requires treatment/medication

13.3 ADVERSE EVENTS REPORTED ON PARTICIPANT DIARY FORMS

Adverse events reported by study participants may include swelling, painful arm(s), difficulty breathing, faintness, flushing, dizziness, weakness, tachycardia, abdominal cramps, or any other systemic manifestation. Symptoms or signs of these events will be monitored and recorded on the daily diary form by study participants according to the following scale:

1 = Mild (barely noticeable and not bothersome)
2 = Moderate (definitely noticeable causing some discomfort)
3 = Severe (needs medical treatment)

Study participants will be instructed to contact a study investigator immediately in the event of any unusual signs or symptoms post injection. Study participants will be instructed to seek medical care at the closest emergency room if a severe adverse reaction occurs during the time periods between office visits.

13.3.1 Adverse Events Guideline

Adverse events can be local and/or generalized and can include itching, swelling, pain, induration, increased heart rate, weakness, faintness, dizziness, nausea/cramps, flu-like symptoms and difficulty breathing. *If any of these events occurs during testing, the testing procedure should be stopped and appropriate action taken to treat the event. Subjects who experience adverse events during testing should not receive additional tests. If such events occur after the subject has left the study site, the subject should be instructed to seek medical attention at the nearest emergency room. The subject is required to complete a daily diary form regarding the time, nature, severity, and outcome of the event. Adverse events shall be graded as mild, moderate, or severe according to the guidelines in Section 13.3.*

13.3.2 Unexpected Adverse Events

Unexpected adverse events are those that characteristically do not occur in response to the intradermal administration of a biological product that is intended to be used as a skin test antigen. Events, such as minor pain, itching, swelling, redness, blistering, necrosis, hives, headache, difficulty breathing, flu-like symptoms, anaphylaxis, are characteristically associated with skin test antigens. Events which are different than those listed above and which are inconsistent with the risk information described in the protocol, investigator's brochure or case report forms should be reported as unexpected adverse experiences.

13.4 DOCUMENTING AND REPORTING ADVERSE EVENTS

At the time of each visit all adverse events either observed or reported, will be documented in the CRF and in the subject's medical records when available. The investigator and clinical monitor team will evaluate each adverse event. Details of any therapeutic measures taken in the event of an AE/SAE will be recorded. Adverse events previously documented in the CRF will be recorded as 'continuing', 'resolved' or 'lost to follow-up', or 'death' at subsequent visits. If an adverse event changes, or advances in quantity or quality, a new record of the event will be initiated

13.4.1 REPORTING ADVERSE EVENTS

1. Serious or unexpected adverse event(s) should be immediately reported to:

   Allermed Laboratories, Inc. ATTN: H. S. Nielsen, Jr. Ph.D.
   Tel: 1.858.292.1060
   Fax: 1.858.292.5934
   Email: *snielsen@allermed.com*

   Human Research Protections Office
   Tel: 1.301.619.2165
   Fax: 1.301.619.7803

   Biomedical Research Institute of America
   Tel: 1.619.282.9997

2. Written reports of serious or unexpected adverse events should be sent to:

   U. S. Army Medical Research and Materiel Command
   ATTN: MCMR-RPH
   504 Scott Street
   Fort Detrick, MD 21702-5012

   FDA MEDWATCH
   5515 Security Lane, Suite 500
   Rockville, MD 20852

13.5 FOLLOW-UP OF ADVERSE EVENTS

The investigator will determine causality of the AE/SAE. This may include additional laboratory testing, follow up visits, and/or histopathological examinations. All adverse events will be followed until resolution

13.6 STOPPING RULES

The occurrence of one serious event (sec. 13.1) shall lead to a review of the study for safety before testing additional subjects. Testing of individual subjects will be stopped if false-positive DTH reactions or sensitization occurs as described in sec. 10.4.2.

14. THE PROTOCOL

14.1 MODIFICATIONS

All amendments to the protocol must be reviewed and approved by the local IRB prior to implementation. Major modifications to the protocol and any modifications that could potentially increase risk to subjects shall be submitted to the local IRB, FDA and USAMRMC ORP HRPO for approval prior to implementation. All other amendments will be submitted with the continuing review report to the USAMRMC ORP HRPO for acceptance.

14.2 DEVIATIONS

Any deviation to the protocol that may have an effect on the safety or rights of the subject or the integrity of the study must be reported to the USAMRMC ORP HRPO as soon as the deviation is identified.

The local IRB shall be notified in writing of such deviation by the principal investigator (in conjunction with the medical monitor) as soon as the deviation is identified.

If either the USAMRMC ORP HRPO or the local IRB considers the deviation significant enough to influence the outcome of the study, the study shall be stopped until agreement is reached by both agencies that the study may continue.

14.3 CONTINUING REVIEW AND FINAL REPORTING

A copy of the local IRB "Approval Notification"; "Continuing Review Report," and "Approved Final Study Report" will be submitted to the USAMRMC ORP HRPO as soon as these documents are available.

14.4 COMPLIANCE

Knowledge of: any inspection/visit or pending inspection/visit by the FDA, HRPO or other government agency concerning clinical investigation or research, the issuance of inspection

reports, FDA Form 483, Warning Letter(s), or actions taken by any regulatory agency including legal or medical actions, and any instance of serious or continuing noncompliance with the regulations or requirements, will be reported immediately to USAMRMC ORP HRPO.

15. ACCESS TO SOURCE DATA/DOCUMENTS

Records relating a subject's participation in the research will remain confidential. Authorized representatives of the U.S. Army Medical Research and Materiel Command, Food and Drug Administration (FDA), the manufacturer of the compounds being tested, members of the HRPO, members of the local Human Use Review Committee, are eligible to review research records as part of their responsibility to protect human subjects in research.

16. QUALITY ASSURANCE

The principal investigator, monitor and investigational staff have participated in clinical trials for other new investigational products. Dr. Donald Brandon is board certified in internal medicine and is experienced in the practice of allergy. He and his staff routinely perform and read skin tests and are familiar with the management of local and systemic reactions that can occur during and after skin testing.

The sponsor has also conducted several clinical trails involving skin testing and has personnel that are familiar with the administration, reading, and recording of immediate and delayed-type skin test reactions. Prior to the start of the study, the sponsor will conduct a meeting with the principal investigator, medical monitor and study staff and review in detail the study protocol and CRF's. In addition, the sponsor will confirm that the investigational site has the necessary equipment and medication immediately available to treat system allergy reactions, including oxygen and adrenalin and short acting corticosteroids. The study site is within 1 mile of a major hospital (Mercy) in case emergency room facilities are needed. For the first several volunteers, the sponsor's representatives will be present during the administration and reading of skin tests with positive control antigens and investigational products to evaluate the accuracy of the skin test procedure, including the administration of reagents, reading of tests and recording results.

Monitoring will be done by persons with appropriate training and certifications. A CRO will not be used. The Sponsor's representative (H.S. Nielsen, Jr. PhD.) will have completed a course on good clinical practices within 12 months of the start date of the study.

17. ETHICS

17.1 RISKS TO SUBJECTS

The risks to volunteers participating in this study are relatively low, whereas the potential benefit to society in the successful development of an effective skin test for *Leishmania* is high. Therefore, on balance, the study stands on solid ethical basis.

17.2 RISKS TO ENVIRONMENT

This study poses no risk to the environment. Waste products include disposable syringes and needles which will be properly disposed of in Sharps containers. The investigational products are non-viable and will not enter the sewage system or be dumped into a landfill.

17.3 COMPENSATION

Volunteers will be paid $50.00 per visit for visits 1-10 and $100.00 for visit 11. The total amount of compensation is intended to cover the cost of transportation and loss of work associated with participation in this study. Payment to volunteer will be made by the business office of the investigational site.

17.4 HUMAN USE OVERSIGHT

Progress on this protocol will be provided to the Human Use Review Committees and to the local IRB's annually, and when appropriate, during the study.

17.5 CONFIDENTIALITY OF DATA

The volunteer's initials and a unique two-digit number will be used to identify the volunteer. All laboratory samples will be identified by the Volunteer's ID number. The Volunteer's ID number will identify all forms used in the study records. All records and computer databases associated with the study will be stored under the supervision and security of the principal investigator. Facsimile copies of the CRFs and Informed Consent will be stored at Allermed under the supervision and security of the Quality Assurance Group.

17.6 MEDICAL CARE

Injury directly related to study procedures and/or investigational products will be paid for by the sponsor. No payment will be made for injuries that are unrelated to study procedures or investigational products or transportation to or from the investigational site.

Because this research is funded by the U.S. Army, the following is available to you in addition to what the Sponsor, Allermed Laboratories, Inc. will provide:

Subjects who are injured as a direct result of this research study are eligible to receive medical care at any Army hospital or clinic free of charge. The Army will not pay for transportation to or from the hospital or clinic. Subjects who pay out-of-pocket expenses for medical care elsewhere for injuries caused by this research study should contact the Principal Investigator. If the issue cannot be resolved, the subject may contact the U.S. Army Medical Research and Materiel Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at 301-619-7663/2221.

18. DATA HANDLING AND RECORDKEEPING

18.1 STATISTICAL ANALYSIS

Repeat doses of 0.1mL LtSTA administered intradermally have the potential to elicit a positive delayed-type hypersensitivity skin test due to the sensitization of lymphocytes in immunocompetent persons who have no previous exposure to *Leishmania.* Repeat skin tests with LtSTA at 30 day intervals can provide information about the sensitizing properties of the antigen. The sample size of this study is too small to provide adequate power for a statistically meaningful outcome. However, due to the exploratory nature of the investigation, it is reasonable to use smaller numbers of subjects to see if the observed rate of sensitization (conversion from a negative to a positive skin test) is too high to be acceptable. This investigation has been designed to evaluate the effect of: 1) repeated skin tests with a fixed dose of LtSTA, and 2) the effect of escalating the antigen dose from 15μg to 30μg to 50μg in separate cohorts. An increase in the number of positive test that parallels an increase in the concentration of the antigen will confirm the sensitizing properties of the antigen. Table 6 shows the 95% one-sided upper confidence limit (UCL) when 10 subjects (N) are skin tested and subjects give a positive result (R). This table shows R from 0 to 3. The data in the table are applicable to all three skin test doses. If R=3 for a given dose at any time point in the study, this dose will be considered unacceptable for further development. Note: twelve (12) subjects will be enrolled in each cohort to allow for two (2) dropouts per cohort. If dropouts do not occur, the first ten (10) subjects enrolled in such cohort will be included in the statistical analysis of data.

Table 6: One-sided 95% upper limit for sensitizing rates

No. of Subjects (N)	Sensitized		One-sided Upper 95% (UCL) for Sensitizing Rate (%)
	R	Observed Rate (%)	
10	0	00.0	25.9
10	1	10.0	39.4
10	2	20.0	50.7
10	3	30.0	60.7

18.2 DISPOSITION OF DATA

According to CFR 21-312.62, the principal investigator will retain all study records for a period of two years following the date a New Drug Application (NDA) is approved for the drug for the indication for which it is being investigated; or if no application is to be filed or if the application is not approved for such indication, until two years after the investigation is discontinued and the FDA is notified. The Sponsors will retain copies of all source documents for a minimum of seven years after FDA is notified that the product is no longer at Allermed. Records will not be destroyed unless a letter is received from the principal investigator specifying that the investigation is discontinued and that the FDA was notified. At that point documents will be shredded and a record kept on site of that action.

19. PUBLICATION POLICY

Results of this study may be presented in scientific forums orally and in written publications in scientific journals. No identifying information for any of the participants in the trial will be included in any presentation of data.

20. RESPONSIBILITIES OF INVESTIGATORS

Donald Brandon, M.D. (Principal Investigator)

A. General Functions:
   1. Understand and follow Good Clinical Practices (GCPs)
   2. Protocol approval
   3. Responsible for overall conduct of clinical trial

B. Specific tasks and responsibilities:
   1. Obtain volunteer's informed Consent
   2. Determine study eligibility based on screening data and the exclusion criteria
   3. Perform or oversees the Volunteer Registration Data Form, Medical History, Physical Exam. Verifies skin test results.
   4. Order tests and blood draws for:
      a. HIV
      b. Hepatitis B
      c. Hepatitis C
      d. Comprehensive Metabolic Panel
      e. CBC Differential
   5. Complete Entrance Exam and Laboratory Test Checklist
   6. Complete Inclusion and Exclusion form and decides on eligibility of volunteer for study
   7. Record adverse events and reports all AE and serious and unexpected adverse events
   8. Assure safety of the volunteers
   9. Supervises the execution of the Case Report forms
   10. Oversees recording all observations and data in the individual subject records
   11. Assure data integrity
   12. Assure volunteer access and follow-up
   13. Assure timely reporting
   14. Assure proper storage of study documents
   15. Control of concomitant medication
   16. Complete Study Termination Record
   17. Complete Drug Accountability Form

William Davis, M.D. (Subinvestigator)

A. General Functions:
   1. Understand and follow Good Clinical Practices (GCPs)

B. Specific tasks and responsibilities:
   2. Obtain volunteer's informed Consent

3. Perform or oversees the Volunteer Registration Data Form, Medical History, Physical Exam. Verifies skin test results.
4. Order tests and blood draws for:
   a. HIV
   b. Hepatitis B
   c. Hepatitis C
   d. Comprehensive Metabolic Panel
   e. CBC Differential
5. Complete Entrance Exam and Laboratory Test Checklist
6. Record adverse events and reports all AE and serious and unexpected adverse events
7. Assure safety of the volunteers
8. Assure data integrity
9. Assure volunteer access and follow-up
10. Assure timely reporting
11. Assure proper storage of study documents
12. Control of concomitant medication

Carolyn Stork (Clinical Research Coordinator)

A. General Functions:
   1. Understand and follow Good Clinical Practices (GCPs)
B. Specific tasks and responsibilities:
   1. Subject recruitment and screening
   2. Informed consent
   3. Vital signs
   4. Phlebotomy, preparation of blood and urine specimens for laboratory analysis
   5. Medical history review
   6. Skin test review
   7. Diary instruction and review
   8. Case report form completion
   9. Query handling
   10. IRB communication
   11. Regulatory document maintenance
   12. SAE reporting

Maria Aceves (Research Assistant)

A. General Functions:
   1. Understand and follow Good Clinical Practices (GCPs)
B. Specific tasks and responsibilities:
   1. Perform and read skin tests
   2. Case report form completion

Bruce Sahba, M.D., F.A.C.G. (Medical Monitor)

A. General Functions:
   1. Understand and follow Good Clinical Practices (GCPs)
B. Specific tasks and responsibilities:

1. Provide medical care and monitor research subjects for conditions that may arise during the conduct of the study
2. Review all serious and unexpected adverse events associated with the protocol
3. Provide an unbiased written report of adverse event (AE) and the relationship of the AE to the test article
4. Indicate agreement or disagreement with the details of the report provided by the study investigator
5. Monitor the safety of volunteers
6. Represent the interest of volunteers and counsels volunteers on ethical questions and possible problems

21. SUPPLEMENTS

- Informed Consent Form
- HIV Testing Consent Form
- Patient's Bill of Rights

22. REFERENCES

1. World Health Organization. Control of the Leishmaniases. 1990. Report No.: 793.

2. Grimaldi G, Tesh R, McMahon-Pratt D. A review of the geographic distribution and epidemiology of leishmaniasis in the New World. American Journal of Tropical Medicine and Hygiene. 1989; 41(6):687-725.

3. Grimaldi G, Tesh R. Leishmaniases of the New World: Current concepts and implications for future research. Clinical Microbiology Reviews. 1993; 6(3):230-250.

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7. Evans T, Teixira M, McAuliffe I, et al. Epidemiology of visceral leishmaniasis in Northeast Brazil. Journal of Infectious Disease. 1992;166:1124-32.

8. Smith D, deShazo R. Delayed hypersensitivity skin testing. . Manual of Clinical Laboratory testing. 4th ed: American Society of Microbiology; 199.

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11. Caldas AJ, Costa JM, Silva AA, Vinhas V, Barral A. Risk factors associated with asymptomatic infection by *Leishmania* chagasi in north-east Brazil. Trans R Soc Trop Med Hyg. 2002;96:21-28.

12. Faber WR, Oskam L, van Gool T, Kroon NC, Knegt-Junk KJ, Hofwegen H, van der Wal AC, Kager PA. Value of diagnostic techniques for cutaneous leishmaniasis. J Am Acad Dermatol. 2003;49:70-74.

13. Follador I, Araujo C, Orge G, Cheng LH, de Carvalho LP, Bacellar O, Almeida RP, Carvalho EM. Immune responses to an inactive vaccine against American cutaneous leishmaniasis together with granulocyte-macrophage colony-stimulating factor. Vaccine. 2002;20:1365-1368.

14. Reed S, Badaro R, Masur H, et al. Selection of a skin test antigen for American visceral leishmaniasis. American Journal of Tropical Medicine and Hygiene. 1986;35:79-85.

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18. Jose FF, da Silva IM, Araujo MI, Almeida RP, Bacellar O, Carvalho EM. Evaluation of the sensitization power of Montenegro skin test. Rev Soc Bras Med Trop. 2001;34:537-542.

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Attachment 3

Protocol Amendment (LtSTA-08 Rev 3A).

BB-IND 11822
Investigational Product: *Leishmania tropica* Skin Test Antigen (LtSTA)

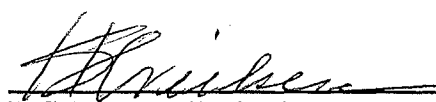
CLINICAL PROTOCOL

Title: A Blinded, Placebo Controlled Study Evaluating Safety, False-Positive Reactions and Sensitizing Properties of 30µg Intracutaneous Doses of *Leishmania tropica* Skin Test Antigen (LtSTA) In Adult Volunteers Without a History of Exposure to *Leishmania spp.*

Phase of Development: II
Study # LtSTA-08 Revision 03A
February 3, 2009
Amendment to LtSTA-08 Rev 03

This study will be performed in compliance with good clinical practice (GCP), including the archiving of essential documents.

Sponsor:

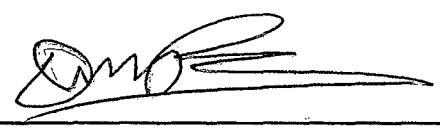


02-03-2009

H.S. Nielsen, Jr., Ph.D.
President
Allermed Laboratories, Inc.

Date

Principal Investigator:



2/3/09

Donald Brandon, M.D.
California Research Foundation
(single study site)
2800 Third Ave
San Diego, CA 92103-6204

Date

TABLE OF CONTENTS

1. GENERAL INFORMATION

USAMMDA Contract Number:	DAMD 17-00-C-0030
IND Number:	BB-IND 11822
Study Number:	LtSTA-08 Rev 03A
Sponsor:	Allermed Laboratories, Inc. 7203 Convoy Ct. San Diego, CA 92111 Phone: 858-292-1060 Attn: Stewart Nielsen, Ph.D.
Principal Investigator:	Donald Brandon, M.D. California Research Foundation 2800 Third Ave, San Diego, CA 92103-6204 Phone: (619) 291-2321
Medical Monitor:	Bruce Sahba, M.D., F.A.C.G. 1855 First Ave Suite 200B San Diego, CA 92101 619-702-2100
Clinical Study Site:	California Research Foundation 2800 Third Ave, San Diego, CA 92103-6204 Phone: (619) 291-2321
Federal-Wide Assurance Number of Clinical Study Site:	FWA00006704
Clinical Laboratory:	Sharp Memorial Hospital Laboratory 7901 Frost Street San Diego, CA. (858)939-3650
Study Duration:	6 months
Required Number of Volunteers:	up to 20
IRB:	Biomed Research Institute of America 3110 Camino del Rio South, Suite A215 San Diego, CA 92108 Phone: (619) 282-9997 Fax: (619)282-9998
Federal-wide Assurance Number of Sponsor:	FWA00011067
Human Research Protection Office (HRPO):	Contact: 504 Scott Street, Fort Detrick, MD 21702-5012 Phone: (301) 619-6239

2. SYNOPSIS

Sponsor: Allermed Laboratories, Inc **Name of Finished Product:** *Leishmania tropica* Skin Test Antigen (LtSTA) **Active Ingredient:** *Leishmania tropica* Promastigote Lysate	BB IND 11822 LtSTA-08, Rev. 03A San Diego	
Title of Study: A Blinded, Placebo Controlled Study Evaluating Safety, False-Positive Reactions and Sensitizing Properties of 30μg Intracutaneous Doses of *Leishmania tropica* Skin Test Antigen (LtSTA) in Adult Volunteers Without a History of Exposure to *Leishmania spp.*		
Investigator(s): Dr. Donald Brandon, M.D.		
Study Center: California Research Foundation 2800 Third Ave, San Diego, CA 92103-6204		
Publication (reference): None		
Study Period: 6 months	Phase of Development: II	

Background Information:

Protocol LtSTA-08 Rev 03 involved skin testing subjects with 15μg, 30μg and 50μg doses of *Leishmania tropica* Skin Test Antigen (LtSTA). Each dose was administered three times at 30 day intervals. The data obtained following this protocol demonstrated that the 50μg dose was sensitizing and, therefore, would not be suitable for use as a skin test antigen which potentially would be administered three times in persons entering and leaving endemic areas.

Sensitization to LtSTA was not observed in subjects receiving the 30μg dose. Therefore, it is considered appropriate to increase the number of subjects in this cohort to obtain more definitive information about the sensitizing capacity of a 30μg dose. Although sensitization was not observed to the 15μg dose, further testing of this dose will not be done under this amendment.

Objectives:

(1) to evaluate the safety of three 30μg/0.1mL doses of LtSTA in healthy adult volunteers who have had no known previous exposure to *Leishmania* parasites;

(2) to provide information on the occurrence of false-positive skin tests to 30μg LtSTA in non-sensitized persons; and

(3) to determine the sensitizing effect of 30μg LtSTA on the outcome of repeat tests at 30 and 60 days.

Methodology:

This trial will involve healthy adult volunteers without previous exposure to *Leishmania* species,. To qualify for enrollment, volunteers must meet the inclusion criteria, pass a medical examination, have acceptable vital signs, and have at least one positive skin test

Sponsor: Allermed Laboratories, Inc	BB IND 11822 LtSTA-08, Rev. 03A San Diego	
Name of Finished Product: *Leishmania tropica* Skin Test Antigen (LtSTA)		
Active Ingredient: *Leishmania tropica* Promastigote Lysate		

to a DTH antigen control to ensure that their cellular immune system is functioning properly.

Volunteers who qualify for enrollment will be skin tested with three 30μg doses of LtSTA. The active drug (LtSTA) will be administered with placebo and saline control on a blinded basis.

Number of Subjects: A maximum of 20 subjects will be enrolled under this amendment of the protocol. The anticipated enrollment is 8-12. However, enrollment of 20 subjects is requested to allow for subjects that drop out, withdraw or are removed from the study.

Diagnosis and Main Criteria for Inclusion:

All volunteers will be subjected to a physical examination and laboratory work-up. The criteria for enrollment are the same as described in Revision 03 of the protocol.

Inclusion Criteria: Male or Female in good health; Age 18 – 60 years; No past history of leishmaniasis or prior participation in a *Leishmania* study; No prior skin test with a *Leishmania* antigen; No occupational, residential, or travel exposure to *Leishmania*; Positive Candin® or Trichophyton skin test (≥ 5 mm induration).

Exclusion Criteria: History of adult atopic dermatitis, contact dermatitis to multiple agents, unexplained urticaria, or asthma; active allergic rhinitis or conjunctivitis; history of allergy or reactions to phenol, polysorbate 80, or glycerol; Medications: currently taking (within the last month) antihistamines or recent history of taking (within the last 1 year) corticosteroids, immunosuppressants; Splenectomy; Active medical disease*; Pregnancy or lactating; Immunization within 4 weeks; History of leishmaniasis; Occupational exposure to *Leishmania;* Prior participation in a *Leishmania* study; Prior skin test with *Leishmania* antigen; Travel history to *Leishmania* endemic areas; Abnormal screening lab results; Keloid scar formation

***Active Medical Disease**: Any active physical or psychiatric condition that may increase the risks associated with participation in the study or interferes with the interpretation of study results. Included chronic medical illnesses are cardiovascular disease, renal insufficiency, chronic respiratory illness, cirrhosis, chronic hepatitis, chronic pancreatitis, chronic diarrhea, malnutrition, malignancy, autoimmune disease, and asthma.

Test Product, Dose and Mode of Administration, Batch Number:

LtSTA is a clear, sterile solution containing the lysate of *L.tropica* promastigotes. The product is standardized by protein content, stabilized with buffered saline and preserved with 0.4% phenol. The dose is 0.1mL administered intradermally in the forearm. The product is provided to the clinical site at the concentration to be tested (30μg/0.1mL) .

Sponsor: Allermed Laboratories, Inc	BB IND 11822 LtSTA-08, Rev. 03A San Diego	
Name of Finished Product: *Leishmania tropica* Skin Test Antigen (LtSTA)		
Active Ingredient: *Leishmania tropica* Promastigote Lysate		

Study Participant Duration of Treatment:

For each study participant the study will last approximately four (4) months. Participants will be skin tested on Visits 3, 6 and 9 of the study. The results of skin tests will be read after 48 hours (± 6 hours) on Visit 4, 7 and 10. A final evaluation is performed on Visit 11, 14 days after Visit 10 (see "Table: Time and Event Schedule).

Reference Therapy, Dose and Mode of Administration, Batch Number:

See dose and administration above.

Criteria for Evaluation:

Efficacy: (1) The absence of induration ≥ 5mm to the first skin test with LtSTA demonstrating that a 30μg dose does not elicit a false-positive DTH reaction; and (2) the absence of induration ≥ 5mm to a second and third skin test with LtSTA demonstrating that prior skin tests with a 30μg dose of LtSTA do not cause sensitization.

Safety: The absence of local and/or systemic reactions to LtSTA including, but not limited to, tenderness or inflammation causing disability/incapacity lasting longer than 24 hours, necrosis at the skin test site, anaphylaxis, and death.

Statistical Methods:

Fisher's exact test will be used to calculate the 95% one-sided upper confidence limit (UCL) for sensitivity.

References:

1. STATA Statistical Software, Release 10, Stata Corp LP, College Station TX.
2. GW Snedecor & WG Cochran, Statistical Methods, 8th Edition, 1989, Iowa State University Press, Ames, Iowa
3. JL Fleiss, Statistical Methods for Rates and Proportions, 2nd Edition, 1981, John Wiley & Sons, New York, NY.

SUMMARY – CONCLUSIONS

Efficacy Results

The efficacy of LtSTA as a skin test antigen depends upon the sensitivity and specificity of the product. This amendment to the study has been designed to determine if a 30μg dose shows non-specific reactivity due to components of the antigen solution and if the product has the ability to sensitize lymphocytes of *Leishmania* naïve persons when administered intradermally. The presence or absence of a local inflammatory response to the first skin test with each of three doses of LtSTA will provide insight on the specificity of the antigen in a naïve population. The local inflammatory response to LtSTA following the first

Sponsor: Allermed Laboratories, Inc	BB IND 11822 LtSTA-08, Rev. 03A San Diego	
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and second repeat skin tests will indicate if the antigen is sensitizing after intradermal administration.

Safety Results:

The safety of LtSTA as a skin test antigen depends upon the type and degree of adverse events associated with its use. Delayed-type hypersensitivity reactions to skin test antigens can range from mild itching, swelling, redness and pain to more severe local reactions, such as necrosis, at the test site. Systemic reactions also can occur including urticaria, gastrointestinal disturbances and respiratory distress leading to anaphylaxis. Study subjects will be monitored for these types of events after the first, second and third skin test. In addition, any change in laboratory findings will be evaluated following the administration of the three dose regime of LtSTA.

CONCLUSION:

The information obtained from this amendment to the study protocol concerning the intradermal administration of LtSTA is critical to the design of a phase III clinical trial and the final labeled use of the product. The objective of the amendment is to determine if the 30μg dose of LtSTA does not cause false-positive reactions in *Leishmania* naïve individuals and does not sensitize recipients after intradermal administration.

3. ABSTRACT

This phase II study will evaluate a 30 μg dose of *Leishmania tropica* Skin Test Antigen (LtSTA) for the ability to elicit positive delayed-type hypersensitivity (DTH) skin test in persons who have not been exposed to the *Leishmania* parasite. This dose will be administered intradermally at 0, 30 and 60 day time points. The test will be read 48 hrs after administration and scored as positive or negative based on the presence or absence of induration > 5mm at the test site. The study will be blinded and placebo controlled to avoid bias in the reading and interpretation of the test results.

Amendment 03A permits the enrollment of additional subjects in the 30μg cohort of Protocol LtSTA-08 Rev 03. The purpose of the amendment is to obtain additional information concerning the sensitizing properties of the 30μg dose in a larger number of naïve individuals. Revision 03 of the protocol provided for the enrollment of twelve (12) subjects in the 30μg cohort. This amendment increases the total count of subjects completing the study to twenty(20) new subjects, with the understanding that the actual number of volunteers enrolled will likely be no more than 8-12.

No additional subjects will be recruited for the 50μg cohort. This dose was found to be sensitizing on the third skin test in two individuals. One individual had a 11.5mm induration response to the 50μg dose and one individual had induration responses that was 4 mm in diameter, suggesting sensitization, even though induration less than 5 mm was considered a negative response.

No evidence of sensitization has been observed with either the 15μg or 30μg doses. However, further study of the 15μg is not considered at this time, since a primary objective of the study (as described in Revision 03) was to determine the highest non-sensitizing dose of the antigen following three skin tests.

In the event that sensitization is not observed with the 30μg dose after administering the antigen three times at monthly intervals to twenty (20) naïve subjects, the 30μg dose will be considered non-sensitizing within the limits specified in the Statistical Section (18.1) of this amendment.

4. LIST OF ABBREVIATIONS AND DEFINITIONS

AE	Adverse event – any untoward medical occurrence in a clinical study subject administered a pharmaceutical product and that does not necessarily have a causal relationship with this treatment.

BCA	Bicinchoninic acid assay – test for protein
CL	Cutaneous leishmaniasis
CRF	Case Report Form
DTH	Delayed-type hypersensitivity
Essential documents	Documents that individually and collectively permit evaluation of the conduct of a study and the quality of the data produced (see section 8 of ICH E6 Guideline for Good Clinical Practice).
GCP	Good Clinical Practices as outlined in ICH E6 Guidance Document
cGMP	Current Good Manufacturing Practice - 21Code of Federal Regulations Part 211
HRPO	Human Research Protections Office
Informed Consent	A process by which a subject voluntarily confirms his/her willingness to participate in a clinical trial, after having been informed of all aspects of the trial. Informed consent is documented by means of a written, signed, and dated informed consent form.
IRB	Institutional Review Board
LtSTA	*Leishmania tropica* Skin Test Antigen
Non-reacting dose	A dose that produces an area of edema < 5mm in diameter
Placebo	Buffered saline solution containing the same ingredients as LtSTA except for the *L.tropica* lysate.
SAE	Serious adverse event
USAMRMC HURO	U. S. Army Medical Research and Materiel Command Human Use Review Office
USAMRMC ORP HRPO	U. S. Army Medical Research and Materiel Command Office of Research Protections, Human Research Protections Office

5. OVERVIEW

5.1 INVESTIGATIONAL PRODUCT

Leishmania tropica Skin Test Antigen (LtSTA), is a sterile injectable microfluidized lysate of *Leishmania tropica* (WR#1063:C1A) promastigotes. The product is heat-treated, filtered, and formulated to a protein concentration of 30μg /0.1mL with 0.85% sodium chloride, 0.4% phenol, 0.01% Tween-80®, and 1% glycerin in phosphate buffer. The antigen is manufactured in compliance with current Good Manufacturing Practices (cGMP) at Allermed Laboratories, San Diego, CA 92111 (see section 10.5 for descriptions of product, placebo, saline and DTH controls).

5.2 PURPOSE OF AMENDMENT TO THE CLINICAL TRIAL

The purpose of this amendment to the clinical trial is three-fold as follows: (1) to evaluate the safety of three 30μg doses of LtSTA in a larger number of healthy adult volunteers with no known previous exposure to *Leishmania* parasites; (2) to provide

information on the occurrence of false-positive skin tests to the initial dose of 30μg LtSTA in *Leishmania* naive persons; and (3) determine the effect of previous skin tests with the 30μg dose of LtSTA on the outcome of repeat tests at 30 and 60 days.

6. INTRODUCTION

Leishmaniasis is a common parasitic disease occurring throughout Africa, Asia, and Latin America. [1-4] The life cycle of the *Leishmania* parasite is complex. The promastigote (form of the parasite found in the insect vector) is normally transmitted to humans by the bite of a female sand fly. Once introduced into a human, promastigotes attach themselves to and invade cells of the mononuclear phagocytic system. After entering macrophages, promastigotes change into amastigotes (the intracellular form of the parasite) and propagate inside a parasitophorous vacuole. The infected macrophage eventually bursts, releasing the amastigotes, which can then infect other target cells. After a blood meal from an infected host, amastigotes are released into the gut of sand flies where they mature into infective promastigotes ready to repeat the cycle.

Infection with *Leishmania* can result in a variety of clinical syndromes, conventionally divided into four major clinical groups: cutaneous, mucocutaneous, diffuse cutaneous, and visceral. The spectrum of disease is so wide that the result of an untreated infection with *Leishmania* ranges from asymptomatic to fatal disease. [5-7] Immunity is largely cell mediated; therefore, tests designed to detect cell-mediated immune responses in exposed individuals are more likely to represent true measures of infection. The use of a skin test to detect delayed-type hypersensitivity (DTH) is a convenient, simple and cost-effective method to assess cell-mediated immune response in humans and can be used for large-scale population surveys. [8] Although there are other methods of detecting cell-mediated immunity, these are difficult to perform, difficult to standardize, and are not practical for screening large numbers of individuals.

Many different skin test preparations have been used in endemic areas in past decades. Most investigators use a locally acquired strain of *Leishmania* to make a crude antigen preparation of whole promastigotes, some form of disrupted promastigotes, or a soluble promastigote antigen. These preparations lack standardization, have unknown sensitivity and specificity, unknown sensitizing capacity, and an undefined dose-response profile between the antigen content and the clinical syndrome or parasite load. In addition, no preparation has been made under a GMP regulatory environment that would allow use in the United States as an investigational new drug or as a commercially available product. Currently, there are several *Leishmania* skin test antigens in use worldwide but none is approved for use in the USA.

7. RISK ASSESSMENT AND PRECAUTIONS

Table: Risk Assessment

Procedure	Risks	Measures to Minimize Risks
Voluntary participation in investigational research project	Breach in confidentiality Anxiety	All laboratory specimens, evaluation forms, reports, and other records will be identified in a manner designed to maintain patient confidentiality. Records will be stored in a secure location with restricted access.
Administration of skin test antigens	Potential risks include: Swelling, painful arm(s), redness at the injection site, pruritis (urge to scratch), difficulty breathing, faintness, flushing, dizziness, weakness, tachycardia, abdominal cramps, itching, induration, nausea, flu-like symptoms, redness, blistering, necrosis, hives, headache, anaphylaxis, vesiculation, tenderness, regional adenopathy or lymphadenopathy (swelling of the lymph nodes), rash, local tissue necrosis, scar formation	Stopping rules are clearly defined. Medical Monitor oversight. Administration of test products by personnel who are properly trained to perform the function. Study subjects will be required to wait in the physician's office for 60 minutes post-administration of the test product. Emergency equipment and personnel will be immediately available at the study site.
Blood Draws	Bruising and bleeding around the site, discomfort, fainting and, rarely, infection	Blood to be drawn by a person trained in phlebotomy using aseptic technique.
Study participation includes HIV, pregnancy and hepatitis testing	Breach in confidentiality Psychological strain if there is a breach in confidentiality. A breach regarding a subject's HIV, pregnancy and/or hepatitis status could have a major impact on both a personal and professional level.	Names and other identifiers will not be directly used on data and specimens to ensure confidentiality of participants. Only investigators will be able to cross match information with participant identity for the purposes of clinical care.

Use of a crude *Leishmania* antigen to elicit DTH in infected individuals was first reported by Montenegro in 1926. [9] Since then many different *Leishmania* skin tests have been given to humans around the world without any reported significant systemic or local reaction. Thousands of doses of various *Leishmania* skin test antigens have been administered for epidemiological surveys and to test seroconversion after administration of vaccines and drugs for leishmaniasis. [10, 11, 12, 13]

The product that will be used in this clinical trial is produced in compliance with cGMPs and will meet requirements for safety, purity, identity and potency prior to use.

Study participants will be instructed to seek medical care at the closest emergency room if a systemic or serious local adverse reaction to the skin test occurs during the time periods between office visits. Participants will be asked to notify the study physician of any serious adverse event as soon as possible.

8. BENEFITS TO SUBJECTS AND COMMUNITY

The study sponsor will compensate subjects for the time lost during the different visits as well as the cost of transportation to the study site. A free copy of the results of the biological tests will be supplied to the subject if requested for his/her medical records. Information regarding any concomitant or intercurrent illnesses detected during the screening will be made available to study subjects. Potential benefits to the community include the availability of an FDA licensed skin test that can be used in the detection of exposure to *Leishmania* parasites.

9. JUSTIFICATION FOR THE ROUTE OF ADMINISTRATION AND DOSAGE

Skin test antigens by nature are given intradermally so that induration can be evaluated. The volume of the injection is 0.1mL. The doses to be administered in this study were chosen based on: (1) the results of previously published studies by others in which antigenic preparations similar to LtSTA were tested in humans; and (2) the results of phase I and II clinical trials conducted by Allermed (see Rev 03 of the Protocol, section 9.1, 9.2).

10. STUDY DESIGN

10.1 GCP STATEMENT

The principal investigator has reviewed this protocol and will conduct the study in full compliance with current Good Clinical Practice Guidelines and FDA regulations as indicated by his signature on the protocol signature page.

10.2 POPULATION TO BE STUDIED

Volunteers, ages 18-60 years, inclusive of gender, race and socioeconomic status, will be enrolled in the study following their expressed consent. They will be recruited from the local community by non-coercive means. Refer to Section 11.1 of the protocol for the recruitment plan.

Each volunteer will be assigned a number upon screening. The numbering system consists of an alpha/numeric combination (Lt08) followed by a two-digit number (01) as a suffix.

As an example, subject number 1 will be assigned subject number Lt08-01. The number that is assigned to a subject will be used for that subject throughout the study.

Subjects will be screened for residential, occupational and travel history to exclude persons that have resided, worked, or traveled in geographic areas that are endemic for *Leishmania* parasites. To qualify for enrollment, volunteers must meet the inclusion and exclusion criteria (see section 11.4 and 11.5), pass a medical examination, have acceptable vital signs, and have at least one positive skin test to a DTH antigen control to ensure that their cellular immune system is functioning properly.

Volunteers who qualify for enrollment will receive the 30μg dose of LtSTA with placebo and saline control on a blinded basis

The minimum legal age for adult enrollment is 18 years. Persons over 60 years of age may have reduced cellular immunity which could potentially affect study results.

10.3 OBJECTIVES

The primary objective of this study is to further evaluate the 30μg dose of LtSTA to determine if it is (1) safe, (2) free of non-specific reactive substances with the potential to cause false-positive reactions, and (3) non-sensitizing, *i.e.*, does not convert negative skin test responders to positive skin test responders.

10.4 PRECAUTIONS AND ENDPONTS

10.4.1 Precautions

1. Subjects should not receive any vaccine 4 weeks prior to the start of the study, during the study, and for at least 2 weeks after LtSTA is administered without discussing it with the principal investigator.

2. Medications (including over-the-counter medicines, such as aspirin, acetaminophen [Panadol, Tylenol] or ibuprofen [Brufen]) should not be taken within two days of starting the study or during the study without discussing the use of these medications with the principal investigator.

10.4.2 Study Endpoints

Safety: The absence of local and/or systemic reactions to LtSTA including, but not limited to, tenderness or inflammation causing disability/incapacity lasting longer than 24 hours, necrosis at the skin test site, anaphylaxis, and death. If the safety endpoints fail, the study will be stopped.

Efficacy: (1) The absence of induration ≥ 5mm to the first skin test with LtSTA demonstrating that a 30μg dose does not elicit a false-positive DTH reaction; and (2) the absence of induration ≥ 5mm to a second and third skin test demonstrating that prior skin tests with a 30μg dose of LtSTA does not cause sensitization. A "non-reacting dose" is

one that produces an area of induration < 5mm. Testing of individual subjects will be stopped if the subject has a positive DTH response to the initial or first repeat skin test.

10.5 INVESTIGATIONAL PRODUCTS AND CONTROLS

10.5.1 Description of LtSTA

LtSTA will be manufactured by Allermed according to approved procedures and under cGMP and supplied at a target protein concentration of 30μg/0.1mL in 2cc vials each containing 1mL of product.

10.5.2 Description of Placebo

The placebo control will be manufactured by Allermed according to approved procedures and under cGMP. The *Leishmania tropica* Skin Test Placebo is formulated to contain all the components of LtSTA except the parasite lysate. The placebo consists of 0.85% sodium chloride, 0.4% phenol, 0.01% Tween-80®, and 1% glycerin in a 25mM phosphate buffer.

10.5.3 Description of the Saline Control

The saline control containing 0.9% sodium chloride (and 0.4% phenol as the preservative) will be manufactured at Allermed.

10.5.4 Description of the Anergy Panel (DTH Controls)

Candin® and Trichophyton allergenic extract (1:500 w/v) will be used as DTH controls for testing the cellular hypersensitivity of study volunteers. Candin® is an FDA approved product manufactured by Allermed Laboratories, Inc. that can be used to detect delayed-type hypersensitivity to the yeast *Candida albicans*. Trichophyton is an FDA approved allergenic extract with DTH properties, manufactured by Allermed Laboratories, Inc. This extract is manufactured from the fungi *Trichophyton rubrum* and *Trichophyton mentagrophytes*, causative agents of dermatophytosis of the hair, nails, and skin. It is expected that between 70% and 80% of trial volunteers with functional cellular immunity will produce a positive DTH response to one or both of the positive control antigens, demonstrating that these individuals are capable of mounting an appropriate cellular immunological response to a DTH antigen. If a volunteer demonstrates a positive response to one of the controls (either Candin® or Trichophyton) then that control shall be used as a positive control for that individual for the remainder of the study. If the volunteer demonstrates a positive response to both controls, Candin® shall be used as the positive control for that individual for the remainder of the study.

10.5.5 Blinding of Investigational Products

LtSTA, placebo and saline vials will be coded. The true identity of each vial will be recorded and enclosed in a sealed envelope and given to the principal investigator with

instructions to open the envelope only if an emergency occurs that requires identification of the investigational products. The positive control (Candin® or Trichophyton) will not be blinded and will be administered as a single injection in the opposite forearm from the three investigational products.

10.5.6 Investigational Product Accountability

All study products and drugs for anaphylaxis will be kept in a secure location at the clinical site. IND materials will be logged into the clinic on arrival from Allermed. A Chain of Custody Form will accompany the investigational products and will become part of the study file. A written log will be maintained and only an authorized person will remove the products from storage for use in the study. The site must contain a monitored refrigerator unit capable of storing the test articles at 2 - 8°C before and after use. A temperature recording devise will be supplied by Allermed. This devise will remain in the storage refrigerator with the product and controls for the length of the study. When the study is complete, the temperature recording devise shall be returned to Allermed.

10.6 PROCEDURES

10.6.1 Testing to be Performed on Blood and Urine

A comprehensive metabolic panel (Table 5), CBC with differential, urinalysis, HIV, Hepatitis B, and Hepatitis C assays will be performed. Pregnancy testing will be performed in the clinic using commercially available pregnancy test strips.

Table 5. Laboratory tests included in the comprehensive metabolic panel.

Albumin	Alkaline Phosphatase	Bilirubin-Total	Potassium
BUN	Calcium	CO2	Chloride
Creatinine	Glucose	GDT (AST)	
GPT (ALT)	Protein-Total	Globulin	
A/G	Bun/Crt	Sodium	

Blood and urine samples will be collected during screening (two 10mL tiger-top tubes for serum, one 5mL lavender-top tube for blood, and one 20mL yellow top container for urine). Approximately 10mL of blood and 20mL of urine will be collected during screening and at the end of study physical examination (one 5mL tiger-top tube for serum, one 5mL lavender-top tube for blood, and one 20mL yellow top container for urine).

Samples will be identified with the subject's ID and placed in plastic bags provided by Sharp Memorial Hospital Laboratory. A requisition form is enclosed in the bag with the sample. A courier employed by Sharp Memorial Hospital Laboratory collects the sample and transports them in a company vehicle to the laboratory. The

samples will then be tested. Once the samples are tested and the report is issued, the samples will be destroyed and will not be used for further research.

All female study participants must either show medical documentation of surgical sterilization or take a urine pregnancy test within the 24 hours before skin testing.

10.6.2 Skin Tests

One-tenth milliliter of each test article will be injected intradermally into the alcohol-cleansed volar surface of the forearm under the supervision of a physician who will have drugs and equipment to treat anaphylaxis immediately available. Skin tests will be placed approximately 2 inches (5cm) apart to avoid overlapping reactions. The diameter of induration and/or erythema will be measured in millimeters at 30 minutes, (to detect immediate IgE hypersensitivity reactions), and at 48 ± 6 hours (induration consistent with DTH) by a combination of the "Sokal" method[30] and the FDA method (Docket No. 94N-0012). Induration and erythema will be measured at 48 ± 6 hours. The indurated border will be outlined as a solid line with a ballpoint pen. Erythema will be outlined with a broken (dotted) line. The tracing will be transferred to paper using transparent tape. The largest diameter and its orthogonal diameter of induration will be measured, and averaged. Erythema will be measured for informational purposes only. The skin test will be placed by a clinical researcher according to the position of the test article shown in the CRF for each subject. Care will be exercised to inject the same 0.1mL volume of the saline control, placebo and LtSTA, so that the 48 hour response to each reagent can be compared.

10.6.2.1 Performing the Test

The placement of the skin tests on the volar surface of the forearm will be predetermined for each study participant. The test is performed by inserting the needlepoint, bevel side up, into the skin at a 15-20 degree angle. The angular distance of penetration should be approximately 1.5mm to pass through the epidermis. This distance can be judged by inserting the needle into the skin until the bevel is no longer visible. Once the needlepoint is in the proper location, 0.1mL of antigen injected into the dermis, maintaining steady pressure on the plunger and slowly advancing the needle tip until the complete volume is injected and the needle is withdrawn. If the test is done correctly, a distinct, sharply defined bleb (5-10mm in diameter) will occur at the injection site. A tiny drop of test antigen at the injection site is not uncommon. The patient may feel a slight burning sensation.

10.6.2.2 Reading the Test

The DTH control skin tests shall be read at 48 ± 6 hours post injection. LtSTA, placebo, and saline control skin tests shall be read after 30 minutes for a wheal/flare response and after 48 ± 6 hours post injection for induration and erythema. The extent of induration is determined by palpating the skin test site with the index finger for firmness. The size of the area of firmness is independent of the erythematous response that may be associated with the reaction. Commonly, DTH reactions have a dense erythematous core surrounded by an area of firmness. The true induration response must include the entire area of firmness, regardless of erythema.

10.6.2.3 Recording the Test

Wheal/flare reactions after 30 minutes shall be reported as mm edema/mm erythema. Skin tests read at 48 ± 6 hours shall be recorded in mm induration using the ballpoint pen method. [19]

10.6.2.4 Measuring and Recording Induration

The area of induration (firmness) shall be outlined with a ballpoint pen. A permanent record of the reaction size shall be made by pressing transparent tape over the tracing and placing the tape on the skin test record. To determine the mean mm induration response, the tracing shall be measured at the greatest induration diameter and its orthogonal diameter and the mean of the two numbers should be calculated.

10.6.2.5 Measures taken to Minimize Bias

A randomization list will be prepared specifying which test article will be placed proximally, distally, or intermediate on the forearms.

10.6.2.6 Breaking the Randomization Code

In the event of an adverse event or unexpected circumstance that requires the identity of a study article (LtSTA or control) the list of coded articles shall be included in the Study Binders.

10.6.2.7 Identification of Source Data

All data will be recorded directly on Case Report Forms (CRFs) with the exception of the clinical laboratory results (computer printouts) which will be added to the CRF file for each volunteer. The volunteer's name on each laboratory result will be masked and replaced with the study number. Study numbers will be assigned at the pre-enrollment testing visit.

10.7 BREAKDOWN BY VISIT TO CLINICAL SITE

10.7.1 Visit #1

1. Complete consent forms, volunteer registration data form, volunteer reported medical history, and medical history review by physician.
2. Check and record vital signs and perform physical exam #1.
3. Perform urine pregnancy test #1 on non-surgically sterilized females.
4. Obtain blood and urine samples
5. Complete Eligibility Record for Study Enrollment
6. Administer Candin® and Trichophyton skin tests per protocol Section 10.6.2.1.
7. Check vital signs after skin testing
8. Complete post procedure volunteer evaluation Visit #1.
9. Give subject daily diary form to record AE until next appointment.

10.7.2 Visit #2 (48 ± 6 hrs after Visit #1)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Read and record skin test results per protocol Sections 10.6.2.2. and 10.6.2.3.
4. Check and record vital signs.
5. Complete post procedure volunteer evaluation Visit #2.
6. Note: Subject must have a positive skin test to Candin® or Trichophyton to continue in the study.

10.7.3 Visit #3 (14 ± 3 days after visit #2)

1. Review laboratory results on blood and urine samples. All values must be within accepted range for subject to continue in the study.
2. Check and record vital signs
3. Perform urine pregnancy test #2 on non-surgically sterilized females.
4. **Administer skin tests with drug products** (LtSTA, Placebo, and Saline) in the left forearm per protocol Section 10.6.2.1. Administer one positive control (either Candin® or Trichophyton) in the right forearm per Section 10.6.2.1.
5. Check and record vital signs 60 minutes post skin testing.
6. Complete post procedure volunteer evaluation Visit #3.
7. Give subject daily diary form to record AE until next appointment.

10.7.4 Visit #4 (48 ± 6 hrs after Visit #3)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Read and record skin test results per protocol Sections 10.6.2.2 and 10.6.2.3.
4. Check and record vital signs.
5. Complete post procedure volunteer evaluation Visit #4.
6. Give daily diary form to subject to record AE until next appointment.

10.7.5 Visit #5 (7 ± 3 days after Visit #4)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Check and record vital signs.

4. Complete volunteer evaluation Visit #5.

10.7.6 Visit #6 (30 ± 7 days after Visit #3)

1. Perform urine pregnancy test #3 on non-surgically sterilized females.
2. Check and record vital signs.
3. **Administer skin tests with drug products** (LtSTA, Placebo, and Saline) in the left forearm per protocol Section 10.6.2.1. Administer one positive control (either Candin® or Trichophyton) in the right forearm per Section 10.6.2.1.
4. Wait 60 minutes after skin testing before checking and recording vital signs.
5. Complete post procedure volunteer evaluation Visit #6.
6. Issue daily diary form to subject to record AE until next appointment.

10.7.7 Visit #7 (48 ± 6 hrs after Visit #6)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Read and record skin test results per protocol Sections 10.6.2.2 and 10.6.2.3.
4. Check and record vital signs.
5. Complete post procedure volunteer evaluation Visit #7.
6. Issue daily diary form to subject to record AE until next appointment

10.7.8 Visit #8 (7 ± 3 days after Visit #7)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Check and record vital signs.
4. Complete volunteer evaluation form.

10.7.9 Visit #9 (30 ± 7 days after Visit #6)

1. Perform urine pregnancy test #4 on non-surgically sterilized females.
2. Check and record vital signs.
3. **Administer skin tests with drug products** (LtSTA, Placebo, and Saline) in the left forearm per protocol Section 10.6.2.1. Administer one positive control (either Candin® or Trichophyton) in the right forearm per Section 10.6.2.1.
4. Wait 60 minutes after skin testing before checking and recording vital signs.
5. Complete post procedure volunteer evaluation Visit #9.
6. Issue daily diary form to subject to record AE until next appointment.

10.7.10 Visit #10 (48 ± 6 hrs after Visit #9)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Read and record skin test results per protocol Sections 10.6.2.2 and 10.6.2.3.
4. Check and record vital signs.
5. Obtain blood and urine samples.
6. Complete post procedure volunteer evaluation Visit #10.
7. Issue daily diary forms to subject to record AE until next appointment.

10.7.11 Visit #11 (14 ± 3 days after Visit #10)

1. Collect and review daily diary form.
2. Report AE on Adverse Events Form.
3. Check and record vital signs.
4. Perform physical exam.
5. Perform urine pregnancy test #5 on non-surgically sterilized females.
6. Review lab results for blood and urine.
7. Complete end of study form. If follow-up is indicated, schedule subject for next appointment.

Table. Time and Event Schedule.

Visit Number	Time	Length of Visit	Events
1	0	2.5 hrs	Consent, history & physical exam, vital signs, blood draw, urine sample, urine pregnancy test (females), administration of DTH control skin tests for anergy. Issue daily diary forms.
2	48 ± 6 hrs after visit 1	30 min	Check vital signs. Read & record DTH control skin tests, review diary, complete study enrollment. Select appropriate DTH control for use during the remainder of the study
3	14 ± 3 days after visit 2	1.5 hrs	Urine pregnancy test (females), vital signs, **administration of LtSTA**, placebo, and saline control skin tests. Post injection vital signs after 60 minutes. Issue new daily diary. Post procedure evaluation.
4	48 ± 6 hrs after visit 3	30 min	Read & record LtSTA and control skin tests results, vital signs, review diary. Post procedure evaluation. Issue daily diary.
5	7 ± 3 days after visit 4	30 min	Review diary, vital signs. Complete volunteer evaluation form.
6	30 ± 7 days after visit 3	1.5 hrs	Urine pregnancy test (females), vital signs, **administration of LtSTA**, placebo, and saline control skin tests. Vital signs after 60 minutes. Issue new diary form
7	48 ± 6 hrs after visit 6	30 min	Read & record LtSTA and control skin tests results, vital signs, review diary, post procedure evaluation. Issue new diary form.
8	7 ± 3 days after visit 7	30 min	Review diary, vital signs. Complete volunteer evaluation form.
9	30 ± 7 days after visit 6	1.5 hrs	Urine pregnancy test (females), vital signs, **administration of LtSTA**, placebo, and saline control skin tests. Vital signs after 60 minutes. Issue new diary form.
10	48 ± 6 hrs after visit 9	30 min	Obtain blood & urine samples. Read & record LtSTA and control skin tests results, vital signs, review diary, post procedure evaluation. Issue new diary form.
11	14 ±3 days after visit 10	1.5 hrs	Review diary, vital signs and physical exam, complete adverse events form, complete volunteer evaluation, complete end of study form.

10.8 MONITORING OVERSIGHT

10.8.1 Subject Safety

Volunteers participating in the study will be monitored for vital signs that conform to study requirement at each visit. Signs and symptoms of adverse reactions during and after the performance of study procedures will be evaluated by study personnel. Daily diaries will be kept by volunteers to record adverse events that occur between scheduled visits. Blood and urine samples will be collected at the beginning and end of the study to determine if metabolic or pathological changes occur that could be related to the investigational products.

10.8.2 Investigational Site

Regular monitoring visits to the medical clinic conducting the protocol will be made by personnel assigned by Allermed to monitor the conduct and progress of the study. Interviews with the principal investigator and investigational staff will be held to evaluate the role of each person involved in the performance of study procedures and record keeping. Some monitoring visits will be made concurrently with visits by study volunteers to observe how the data required by the protocol is being obtained.

10.8.3 Source Documents

CRF's will serve as source documents unless information related to the study or well being of study volunteers can not be adequately captured on the forms provided. All documents relating to the study procedures and any documents that result from actions relating to the study will be archived by the principal investigator and, where appropriate, copies will be kept by Allermed in a secure location.

10.8.4 Analysis of Data

Data obtained from the study will be reviewed and analyzed by Allermed personnel or by consultants employed by Allermed. Personal information of study volunteers will be maintained on a confidential basis in compliance with the laws and regulations pertaining to patient privacy.

11. SELECTION, WITHDRAWAL AND REPLACEMENT OF SUBJECTS

11.1 RECRUITMENT PLAN

Volunteers will be recruited from the patient data base of the clinical site. If other recruiting measures are needed, such as placing an advertisement in the local newspaper or by posting or distributing flyers in public areas, such advertisement or flyers will be sent to the IRB for review and approval. Refer to Section 10.2 for specifics of the population to be studied.

11.2 INFORMED CONSENT PROCESS

Study volunteers will be consented by either the principal investigator or a clinical research associate. This will take place at the study site and volunteers will be given sufficient time to make an informed decision about participation in the clinical trial, including the ability to

take the consent forms home for review. Questions will be answered by either the principal investigator, clinical research associate, or an agent of the sponsor. Additionally, study recruits will be given the IRB contact information for questions relating to their rights as a research subject. As indicated in the consent form, information learned during the course of the trial which may affect a volunteer's decision to participate will be explained to all study volunteers.

Volunteers will be informed that the principal investigator may terminate their participation in the study without regard to their consent. The circumstances under which this may occur include a volunteer exhibiting behavior that may bias the study, demonstrating behavior indicating mental or emotional unfitness to continue the study, or nonconformance to the protocol requirements. In all cases both the principal investigator and medical monitor will make a joint decision as to whether the volunteer's participation in the study will be terminated.

11.3 EVALUATION BEFORE ENTRY

Each volunteer will sign an informed consent. Volunteers will undergo a medical history screening with special attention to a history of asthma, atopic skin disease and other allergic diseases. This will be followed by physical examination, and screening laboratory tests, including urine pregnancy test (if non-surgically sterilized female), and serological studies for hepatitis B, hepatitis C, and HIV infection. Female volunteers who are not surgically sterile will be asked to take a urine pregnancy test within 24 hours before anergy skin tests and LtSTA administration. All volunteers will sign an informed consent for HIV antibody testing (attached) and will undergo DTH (anergy) testing with Candin® and Trichophyton allergenic extract (1:500 w/v). Induration equal to or exceeding 5mm for at least one of the DTH control antigens will be evidence of normal delayed hypersensitivity.

11.4 INCLUSION CRITERIA

- Male or Female in good health
- Age 18 – 60 years
- No past history of leishmaniasis or prior participation in a *Leishmania* study
- No prior skin test with a *Leishmania* antigen
- No occupational, residential, or travel exposure to *Leishmania*
- Positive Candin® or Trichophyton skin test (≥ 5mm induration)

11.5 EXCLUSION CRITERIA

- History of adult atopic dermatitis, contact dermatitis to multiple agents, unexplained urticaria, or asthma
- Active allergic rhinitis or conjunctivitis
- History of allergy or reactions to phenol, polysorbate 80, or glycerol
- Medications: Currently taking (within the last month) antihistamines or recent history of taking (within the last 1 year) corticosteroids, immunosuppressants.
- Splenectomy
- Active medical disease *
- Pregnancy or lactating
- Immunization within 4 weeks
- History of leishmaniasis
- Occupational exposure to *Leishmania*
- Prior participation in a *Leishmania* study
- Prior skin test with *Leishmania* antigen
- Travel history to *Leishmania* endemic areas
- Abnormal screening lab results
- Keloid scar formation

***Active Medical Disease**: Any active physical or psychiatric condition that may increase the risks associated with participation in the study or interferes with the interpretation of study results. Included chronic medical illnesses are: cardiovascular disease, renal insufficiency, chronic respiratory illness, cirrhosis, chronic hepatitis, chronic pancreatitis, chronic diarrhea, malnutrition, malignancy, autoimmune disease, and asthma.

Pregnancy prevention: Females will be instructed to abstain from sexual relations or practice a method of birth control during the study and two weeks following the end of the study. They will further be instructed that except for surgical removal of the uterus, birth control methods such as the use of condoms, a diaphragm or a cervical cap, birth control pills, IUD, or sperm killing products are not totally effective in preventing pregnancy.

11.6 WITHDRAWAL CRITERIA

Volunteers will be allowed to withdraw from the study at any time without prejudice or loss of benefits to which they are entitled. Volunteers may be removed from the study by the principal investigator or the medical monitor should their continued participation be injurious to their health and well being at any time. The Human Research Protections Office (HRPO) and the local IRB will be notified whenever a subject is withdrawn from the study. Volunteers removed from the study subsequent to receiving the skin test will have a follow up visit after 48 hours ± 6 hours. Drop-out volunteers will be replaced. Although this study is of a relatively short duration, the dropout rate is expected to be very low.

11.7 REPLACEMENT CRITERIA

Volunteers meeting all inclusion/exclusion criteria will be enrolled as needed to replace drop-outs.

11.7.1 Non-Qualifying Volunteers

Persons who sign the informed consent, but who do not qualify for enrollment in the study due to: (1) their failure to meet all inclusion/exclusion criteria or, (2) other documented conditions considered sufficient by the principal investigator or medical monitor to warrant the exclusion of the volunteer, will be informed that they cannot participate in the study. The reason for exclusion will be explained to the volunteer and appropriate recommendations will be provided by the principal investigator in consultation with the medical monitor. All records, laboratory reports, etc., relating to the enrollment process for the volunteer shall be kept and maintained with other study documents.

11.7.2 Volunteers with Concomitant or Intercurrent Illness Detected during Screening

Persons with concomitant or intercurrent illness that is detected during enrollment screening will be excluded from participation in the study. These individuals will be considered non-qualifying volunteers. The procedures that will be followed are the same as those described above for Non-Qualifying Volunteers.

12. MEDICATIONS

The drugs not permitted are described in the exclusion criteria Section 11.5. In addition, study volunteers will be encouraged to avoid all new prescription and over the counter medications during the 7 days after the LtSTA application, and will be asked to contact a study physician immediately should any new medications be prescribed.

13. ASSESSMENT OF SAFETY

Safety of the skin test is demonstrated by the absence of adverse events following skin tests with the investigational products. Adverse reactions will be classified as immediate, generalized/systemic, or local, and the reactions will be graded on a scale including mild, moderate, and severe. Occurrence of adverse events will lead to a review of the study for safety before testing any further subjects per the "Stopping Rules" in Section 13.6.

An adverse event temporally related to participation in the study should be documented whether or not considered to be related to the test article. This definition includes intercurrent illnesses and injuries, and exacerbation of preexisting conditions.

13.1 SERIOUS ADVERSE EVENTS (SAE)

A serious adverse event is any untoward medical occurrence that:

- Results in death
- Is life-threatening [A life threatening event is an event which presents the risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death had it been more severe.]
- Requires in-patient hospitalization
- Results in persistent or significant disability/incapacity [A disabling/ incapacitating adverse event is any event which may result in a substantial disruption of the volunteer's ability to carry out normal life functions. This definition is not intended to include minor cases of headache, nausea, vomiting, diarrhea, influenza, rhinorrhea, lacrimation or accidental trauma, such as a sprained ankle.]
- Results in a congenital anomaly
- Is serious based on the medical judgment of the principal investigator and/or the medical monitor or consulting physician

13.2 ADVERSE EVENTS REPORTED BY INVESTIGATOR OR PHYSICIAN

13.2.1 Immediate Reactions

The following reactions occurring within 60 minutes after injection of the investigational product will be considered mild, moderate, or severe as judged by a physician:

- Subjective feeling of intense anxiety or panic
- Flushing and sweating
- Onset of nausea, vomiting, cramps or diarrhea
- Onset of pruritus in skin or mucous membranes
- Onset of urticaria or angioedema
- Acute onset of rhinorrhea, coughing, wheezing, or dyspnea
- Progressive signs of an anaphylactic reaction
- Hypertension or hypotension

13.2.2 Generalized/Systemic reactions

The following reactions occurring between 30 minutes and 24 hours following injection of the investigational product will be considered mild, moderate, or severe as judged by a physician:

- Fever (temperature greater than 100° F) with or without chills
- Nausea, vomiting, abdominal cramps, diarrhea (acute onset of more than 2 watery stools within a 24 hour period)
- Wheezing or dyspnea
- Uticaria or angioedema

13.2.3 Local reactions

The following reactions occurring within two weeks following injection of the investigational product will be considered mild, moderate, or severe as judged by a physician.

Reactions generally considered mild

- Itching, burning, mild discomfort
- Erythema/edema < 30mm

Reactions generally considered moderate

- Noticeable discomfort, but not compromising limb function
- Erythema/edema ≥ 30mm and < 80mm
- Blistering at test site

Reactions considered severe

- Pain causing decreased motion and use of the limb
- Erythema/edema > 80mm
- Necrosis at the test site
- Requires treatment/medication

13.3 ADVERSE EVENTS REPORTED ON PARTICIPANT DIARY FORMS

Adverse events reported by study participants may include swelling, painful arm(s), difficulty breathing, faintness, flushing, dizziness, weakness, tachycardia, abdominal cramps, or any other systemic manifestation. Symptoms or signs of these events will be monitored and recorded on the daily diary form by study participants according to the following scale:

1 = Mild (barely noticeable and not bothersome)
2 = Moderate (definitely noticeable causing some discomfort)
3 = Severe (needs medical treatment)

Study participants will be instructed to contact a study investigator immediately in the event of any unusual signs or symptoms post injection. Study participants will be instructed to seek medical care at the closest emergency room if a severe adverse reaction occurs during the time periods between office visits.

13.3.1 Adverse Events Guideline

Adverse events can be local and/or generalized and can include itching, swelling, pain, induration, increased heart rate, weakness, faintness, dizziness, nausea/cramps, flu-like symptoms and difficulty breathing. *If any of these events occurs during testing, the testing procedure should be stopped and appropriate action taken to treat the event. Subjects who experience adverse events during testing should not receive additional tests. If such events occur after the subject has left the study site, the subject should be instructed to seek medical attention at the nearest emergency room. The subject is required to complete a daily diary form regarding the time, nature, severity, and outcome of the event. Adverse events shall be graded as mild, moderate, or severe according to the guidelines in Section 13.3.*

13.3.2 Unexpected Adverse Events

Unexpected adverse events are those that characteristically do not occur in response to the intradermal administration of a biological product that is intended to be used as a skin test antigen. Events, such as minor pain, itching, swelling, redness, blistering, necrosis, hives, headache, difficulty breathing, flu-like symptoms, anaphylaxis, are characteristically associated with skin test antigens. Events which are different than those listed above and which are inconsistent with the risk information described in the protocol, investigator's brochure or case report forms should be reported as unexpected adverse experiences.

13.4 DOCUMENTING AND REPORTING ADVERSE EVENTS

At the time of each visit all adverse events either observed or reported, will be documented in the CRF and in the subject's medical records when available. The investigator and clinical monitor team will evaluate each adverse event. Details of any therapeutic measures taken in the event of an AE/SAE will be recorded. Adverse events previously documented in the CRF will be recorded as 'continuing', 'resolved' or 'lost to follow-up', or 'death' at subsequent visits. If an adverse event changes, or advances in quantity or quality, a new record of the event will be initiated

13.4.1 REPORTING ADVERSE EVENTS

1. Serious or unexpected adverse event(s) should be immediately reported to:

   Allermed Laboratories, Inc. ATTN: H. S. Nielsen, Jr. Ph.D.
   Tel: 1.858.292.1060
   Fax: 1.858.292.5934
   Email: *snielsen@allermed.com*

   Human Research Protections Office
   Tel: 1.301.619.2165
   Fax: 1.301.619.7803

   Biomedical Research Institute of America
   Tel: 1.619.282.9997

2. Written reports of serious or unexpected adverse events should be sent to:

   U. S. Army Medical Research and Materiel Command
   ATTN: MCMR-RPH
   504 Scott Street
   Fort Detrick, MD 21702-5012

   FDA MEDWATCH
   5515 Security Lane, Suite 500
   Rockville, MD 20852

13.5 FOLLOW-UP OF ADVERSE EVENTS

The investigator will determine causality of the AE/SAE. This may include additional laboratory testing, follow up visits, and/or histopathological examinations. All adverse events will be followed until resolution

13.6 STOPPING RULES

The occurrence of one serious event (sec. 13.1) shall lead to a review of the study for safety before testing additional subjects. Testing of individual subjects will be stopped if false-positive DTH reactions or sensitization occurs as described in sec. 10.4.2.

14. THE PROTOCOL

14.1 MODIFICATIONS

All amendments to the protocol must be reviewed and approved by the local IRB prior to implementation. Major modifications to the protocol and any modifications that could potentially increase risk to subjects shall be submitted to the local IRB, FDA and USAMRMC ORP HRPO for approval prior to implementation. All other amendments will be submitted with the continuing review report to the USAMRMC ORP HRPO for acceptance.

14.2 DEVIATIONS

Any deviation to the protocol that may have an effect on the safety or rights of the subject or the integrity of the study must be reported to the USAMRMC ORP HRPO as soon as the deviation is identified.

The local IRB shall be notified in writing of such deviation by the principal investigator (in conjunction with the medical monitor) as soon as the deviation is identified.

If either the USAMRMC ORP HRPO or the local IRB considers the deviation significant enough to influence the outcome of the study, the study shall be stopped until agreement is reached by both agencies that the study may continue.

14.3 CONTINUING REVIEW AND FINAL REPORTING

A copy of the local IRB "Approval Notification", "Continuing Review Report," and "Approved Final Study Report" will be submitted to the USAMRMC ORP HRPO as soon as these documents are available.

14.4 COMPLIANCE

Knowledge of: any inspection/visit or pending inspection/visit by the FDA, HRPO or other government agency concerning clinical investigation or research, the issuance of inspection

reports, FDA Form 483, Warning Letter(s), or actions taken by any regulatory agency including legal or medical actions, and any instance of serious or continuing noncompliance with the regulations or requirements, will be reported immediately to USAMRMC ORP HRPO.

15. ACCESS TO SOURCE DATA/DOCUMENTS

Records relating a subject's participation in the research will remain confidential. Authorized representatives of the U.S. Army Medical Research and Materiel Command, Food and Drug Administration (FDA), the manufacturer of the compounds being tested, members of the HRPO, members of the local Human Use Review Committee, are eligible to review research records as part of their responsibility to protect human subjects in research.

16. QUALITY ASSURANCE

The principal investigator, monitor and investigational staff have participated in clinical trials for other new investigational products. Dr. Donald Brandon is board certified in internal medicine and is experienced in the practice of allergy. He and his staff routinely perform and read skin tests and are familiar with the management of local and systemic reactions that can occur during and after skin testing.

The sponsor has also conducted several clinical trails involving skin testing and has personnel that are familiar with the administration, reading, and recording of immediate and delayed-type skin test reactions. Prior to the start of the study, the sponsor will conduct a meeting with the principal investigator, medical monitor and study staff and review in detail the study protocol and CRF's. In addition, the sponsor will confirm that the investigational site has the necessary equipment and medication immediately available to treat system allergy reactions, including oxygen and adrenalin and short acting corticosteroids. The study site is within 1 mile of a major hospital (Mercy) in case emergency room facilities are needed. For the first several volunteers, the sponsor's representatives will be present during the administration and reading of skin tests with positive control antigens and investigational products to evaluate the accuracy of the skin test procedure, including the administration of reagents, reading of tests and recording results.

Monitoring will be done by persons with appropriate training and certifications. A CRO will not be used. The Sponsor's representative (H.S. Nielsen, Jr. PhD.) will have completed a course on good clinical practices within 12 months of the start date of the study.

17. ETHICS

17.1 RISKS TO SUBJECTS

The risks to volunteers participating in this study are relatively low, whereas the potential benefit to society in the successful development of an effective skin test for *Leishmania* is high. Therefore, on balance, the study stands on solid ethical basis.

17.2 RISKS TO ENVIRONMENT

This study poses no risk to the environment. Waste products include disposable syringes and needles which will be properly disposed of in Sharps containers. The investigational products are non-viable and will not enter the sewage system or be dumped into a landfill.

17.3 COMPENSATION

Volunteers will be paid $50.00 per visit for visits 1-10 and $100.00 for visit 11. The total amount of compensation is intended to cover the cost of transportation and loss of work associated with participation in this study. Payment to volunteer will be made by the business office of the investigational site.

17.4 HUMAN USE OVERSIGHT

Progress on this protocol will be provided to the Human Use Review Committees and to the local IRB's annually, and when appropriate, during the study.

17.5 CONFIDENTIALITY OF DATA

The volunteer's initials and a unique two-digit number will be used to identify the volunteer. All laboratory samples will be identified by the Volunteer's ID number. The Volunteer's ID number will identify all forms used in the study records. All records and computer databases associated with the study will be stored under the supervision and security of the principal investigator. Facsimile copies of the CRFs and Informed Consent will be stored at Allermed under the supervision and security of the Quality Assurance Group.

17.6 MEDICAL CARE

Injury directly related to study procedures and/or investigational products will be paid for by the sponsor. No payment will be made for injuries that are unrelated to study procedures or investigational products or transportation to or from the investigational site.

Because this research is funded by the U.S. Army, the following is available to you in addition to what the Sponsor, Allermed Laboratories, Inc. will provide:

Subjects who are injured as a direct result of this research study are eligible to receive medical care at any Army hospital or clinic free of charge. The Army will not pay for transportation to or from the hospital or clinic. Subjects who pay out-of-pocket expenses for medical care elsewhere for injuries caused by this research study should contact the Principal Investigator. If the issue cannot be resolved, the subject may contact the U.S. Army Medical Research and Materiel Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at 301-619-7663/2221.

18. DATA HANDLING AND RECORDKEEPING

18.1 STATISTICAL ANALYSIS

Repeat doses of 0.1mL LtSTA administered intradermally have the potential to elicit a positive delayed-type hypersensitivity skin test due to the sensitization of lymphocytes in immunocompetent persons who have no previous exposure to *Leishmania*. Repeat skin tests with LtSTA at 30 day intervals can provide information about the sensitizing properties of the antigen. The sample size of this study is too small to provide adequate power for a statistically meaningful outcome. However, due to the exploratory nature of the investigation, it is reasonable to use smaller numbers of subjects to see if the observed rate of sensitization (conversion from a negative to a positive skin test) is too high to be acceptable. This amended investigation has been designed to evaluate the effect of: repeated skin tests with a 30μg dose of LtSTATable 6 shows the 95% one-sided upper confidence limit (UCL) when 20 subjects (N) are skin tested and subjects give a positive result (R). This table shows R from 0 to 3.

Table 6: One-sided 95% upper limit for sensitizing rates

No. of Subjects (N)	Sensitized		One-sided Upper 95% (UCL) for Sensitizing Rate (%)
	R	Observed Rate (%)	
20	0	00.0	13.9
20	1	5.0	21.6
20	2	10.0	28.3
20	3	15.0	34.4

18.2 DISPOSITION OF DATA

According to CFR 21-312.62, the principal investigator will retain all study records for a period of two years following the date a New Drug Application (NDA) is approved for the drug for the indication for which it is being investigated; or if no application is to be filed or if the application is not approved for such indication, until two years after the investigation is discontinued and the FDA is notified. The Sponsors will retain copies of all source documents for a minimum of seven years after FDA is notified that the product is no longer at Allermed. Records will not be destroyed unless a letter is received from the principal investigator specifying that the investigation is discontinued and that the FDA was notified. At that point documents will be shredded and a record kept on site of that action.

19. PUBLICATION POLICY

Results of this study may be presented in scientific forums orally and in written publications in scientific journals. No identifying information for any of the participants in the trial will be included in any presentation of data.

20. RESPONSIBILITIES OF INVESTIGATORS

Donald Brandon, M.D. (Principal Investigator)

A. General Functions:
   1. Understand and follow Good Clinical Practices (GCPs)
   2. Protocol approval
   3. Responsible for overall conduct of clinical trial

B. Specific tasks and responsibilities:
   1. Obtain volunteer's informed Consent
   2. Determine study eligibility based on screening data and the exclusion criteria
   3. Perform or oversees the Volunteer Registration Data Form, Medical History, Physical Exam. Verifies skin test results.
   4. Order tests and blood draws for:
      a. HIV
      b. Hepatitis B
      c. Hepatitis C
      d. Comprehensive Metabolic Panel
      e. CBC Differential
   5. Complete Entrance Exam and Laboratory Test Checklist
   6. Complete Inclusion and Exclusion form and decides on eligibility of volunteer for study
   7. Record adverse events and reports all AE and serious and unexpected adverse events
   8. Assure safety of the volunteers
   9. Supervises the execution of the Case Report forms
   10. Oversees recording all observations and data in the individual subject records
   11. Assure data integrity
   12. Assure volunteer access and follow-up
   13. Assure timely reporting
   14. Assure proper storage of study documents
   15. Control of concomitant medication
   16. Complete Study Termination Record
   17. Complete Drug Accountability Form

William Davis, M.D. (Subinvestigator)

A. General Functions:
   1. Understand and follow Good Clinical Practices (GCPs)

B. Specific tasks and responsibilities:
   2. Obtain volunteer's informed Consent
   3. Perform or oversees the Volunteer Registration Data Form, Medical History, Physical Exam. Verifies skin test results.
   4. Order tests and blood draws for:
      a. HIV
      b. Hepatitis B
      c. Hepatitis C
      d. Comprehensive Metabolic Panel

e. CBC Differential

5. Complete Entrance Exam and Laboratory Test Checklist
6. Record adverse events and reports all AE and serious and unexpected adverse events
7. Assure safety of the volunteers
8. Assure data integrity
9. Assure volunteer access and follow-up
10. Assure timely reporting
11. Assure proper storage of study documents
12. Control of concomitant medication

Carolyn Stork (Clinical Research Coordinator)

A. General Functions:
   1. Understand and follow Good Clinical Practices (GCPs)

B. Specific tasks and responsibilities:
   1. Subject recruitment and screening
   2. Informed consent
   3. Vital signs
   4. Phlebotomy, preparation of blood and urine specimens for laboratory analysis
   5. Medical history review
   6. Skin test review
   7. Diary instruction and review
   8. Case report form completion
   9. Query handling
   10. IRB communication
   11. Regulatory document maintenance
   12. SAE reporting

Maria Aceves (Research Assistant)

A. General Functions:
   1. Understand and follow Good Clinical Practices (GCPs)

B. Specific tasks and responsibilities:
   1. Perform and read skin tests
   2. Case report form completion

Bruce Sahba, M.D., F.A.C.G. (Medical Monitor)

A. General Functions:
   1. Understand and follow Good Clinical Practices (GCPs)

B. Specific tasks and responsibilities:
   1. Provide medical care and monitor research subjects for conditions that may arise during the conduct of the study
   2. Review all serious and unexpected adverse events associated with the protocol
   3. Provide an unbiased written report of adverse event (AE) and the relationship of the AE to the test article
   4. Indicate agreement or disagreement with the details of the report provided by the study investigator

5. Monitor the safety of volunteers
6. Represent the interest of volunteers and counsels volunteers on ethical questions and possible problems

21. SUPPLEMENTS

- Informed Consent Form
- HIV Testing Consent Form
- Patient's Bill of Rights

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Attachment 4

Relative Potency Test Method Validation Protocol.

Relative Potency Test Method (RPTM) for Evaluating the Potency of *Leishmania tropica* Skin Test Antigen (LtSTA) Lots with Respect to a LtSTA Internal Reference Standard (LRS)

BB-IND 11822

Sponsor
Allermed Laboratories, Inc.
7203 Convoy Court
San Diego, CA 92111

September 10, 2008

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DP# 0018, DP0018F1, DP0018F2

Relative Potency Test Method (RPTM) for Evaluating the Potency of *Leishmania tropica* Skin Test Antigen (LtSTA) Lotswith Respect to a LtSTA Internal Reference Standard (LRS)

1.0 INTRODUCTION

Leishmania tropica Skin Test Antigen (LtSTA) is used to detect delayed-type hypersensitivity to *Leishmania* parasites. The recommended dose for this product is 30 μg per 0.1 mL, as determined by clinical trials conducted by Allermed Laboratories. It has been established that an Internal Reference Standard (LRS) prepared by Allermed has a potency of 30 μg per 0.1 mL. Allermed has developed a procedure that insures the production of manufactured lots of LtSTA that are comparable in potency to this reference standard. It is believed that a procedure based on the potency value of a production lot relative to the potency of an internal reference standard of known and acceptable potency will minimize the variability within assays performed. It is recognized that variation in this test can result from the dilutions of the reagents, the guinea pigs used in the test, and/or the technicians performing the test, but because the effects are exerted on both the reference and production lots in parallel, the impact of these variables on the results of the test is minimized, if not completely eliminated.

2.0 RELATIVE POTENCY TEST METHOD

A LTSTA lot is tested in parallel with the reference lot (LRS) to estimate the potency of the LTSTA lot with respect to the LRS. The assigned potency of LRS is 30 μg per 0.1 mL.

2.1 Dilution Scheme

Four dilutions of LRS and a LTSTA production lot are prepared. Starting with the undiluted strength (1:1), three two-fold serial dilutions of 1:2, 1:4 and 1:8 are made as follows:

Dilution 1 = 1:1 (300 µg/mL)

Dilution 2 = 1:2 (150 µg/mL)

Dilution 3 = 1:4 (75 µg/mL)

Dilution 4 = 1:8 (37.5 µg/mL)

2.2 Test Model

Four highly sensitized guinea pigs are selected for each relative potency assay. Guinea pigs are sensitized according to DP# 0018 included in the Appendix.

2.2.1 Safety Requirements

Needles shall never be recapped unless it is necessary to maintain sterility of the needle. Potentially biohazardous material is handled with the appropriate precautions.

2.2.2 Examination of Guinea pigs

All guinea pigs undergoing sensitization or included in the potency testing pool are visually examined and weighed every other day except weekends and holidays. Only animals that are in overt good health should be used in the assay.

2.2.3 Guinea Pig Identification

Animals are individually identified prior to sensitization. Identification is done by tattoo. Manufacturer's instructions are followed for applying tattoos.

2.2.4 Preparation of Antigen/Adjuvant Emulsion

Unless otherwise specified the adjuvant used for the initial sensitization injection is Complete Freund's adjuvant (CFA). Boosts may be given with either Incomplete Freund's adjuvant (IFA) or administered as antigen alone. This information is documented in DP 0018F2 (Appendix).

Equal amounts of adjuvant (CFA or IFA) and antigen are drawn into two separate syringes (the adjuvant in one and antigen in the other). The total liquid volume in each syringe should not exceed ½ the total syringe volume. Note: The final mixture is designed to contain approximately 50% adjuvant.

Needles are aseptically removed from each syringe and the syringes are attached to a male adapter on a 3-way stopcock (Baxter #2C6240 or equivalent). The handle is adjusted on the stopcock so that only the flow path between the two syringes is open. The plunger of one syringe is depressed such that all of the fluid flows into the second syringe. The material is emulsified by expelling the liquid back and forth through the stopcock and into the empty syringe for at least twenty cycles. One cycle equals movement from one syringe to the other and then back to the first syringe.

Once emulsified, the full syringe is removed from the stopcock and a sterile needle is placed on the luerlock tip. The emulsion is injected into a sterile empty vial. Emulsified antigen/adjuvant mixtures must be used within 3 hours. The vial is labeled with the name of the material, the adjuvant used, the date and time the emulsion was made and expiration time (3 hours after the emulsion was made).

2.2.5 Injections

For sensitization, a maximum of 0.6 mL can be given in a total of 4 subcutaneous injections per animal. The maximum allowable injection volume is 0.2 mL per site. An appropriate anesthetic is administered to guinea pigs just prior to injection of the adjuvant emulsion. Table 1 shows the acceptable injection sites. Injection volume per animal and injection sites are recorded in DP 0018F2 (Appendix).

Table 1. Injection Sites and Volumes for Sensitization

Injection Site	Maximum Volume Allowed
Neck at nape	0.2 mL
Rump	0.2 mL per side
Inguinal Areas (groin)	0.1 mL per area

Animals are weighed and the appropriate volume of anesthetic is determined (Table 2). If ketamine is used alone, the maximum dose should be given to each animal. If ketamine is used in combination with xylazine, the initial dose may be 50-100% of the maximum dose. If less than a maximum dose is administered in the initial injection, a subsequent injection of anesthetic may be given such that the total amount of anesthetic administered to the animal is equal to the maximum dose. The maximum dose per animal is never exceeded in a single 24-hour period.

Anesthetic shall be administered by intramuscular injection. Dosage are documented in DP 0018F2 (Appendix) and the total volume of ketamine used is recorded in the Controlled Drug Disposition Record.

Table 2. Maximum Anesthetic Volume per Animal Body Weight

Anesthetic	Maximum Dose/Animal
Ketamine	40 mg/Kg body weight
Ketamine/Xylazine	40 mg/Kg body weight (Ketamine) 5 mg/Kg body weight (Xylazine)

Syringes are prepared with 21 gauge needles (smaller bore needles may also be used) containing the appropriate injection volume of antigen or antigen/adjuvant emulsion.

2.2.6 Injection Timing

Animals are given at least a 14-day rest period between sensitization injections and potency tests.

3.0 RELATIVE POTENCY TEST PROCEDURE

Animals may be tested for sensitization 14 days after the 1st injection by skin testing them with the LRS. A positive response (≥ 5mm) to the LRS is evidence of sensitization. Once sensitized, animals may be used for potency testing.

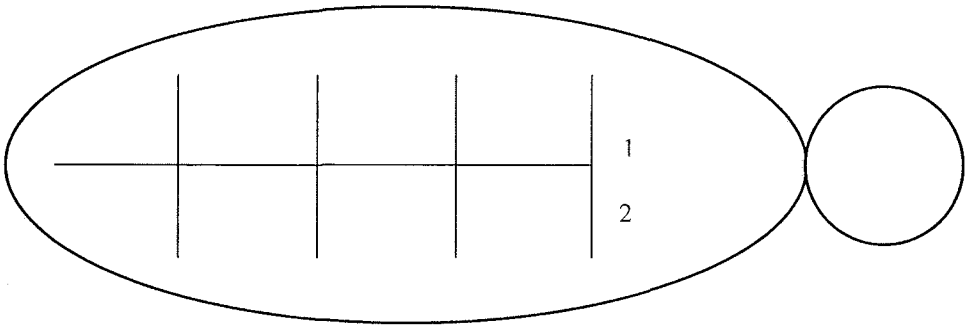
3.1 Performing the Test

Anesthetic shall be administered to all guinea pigs just prior to skin testing. Necessary information is recorded in DP 0018F2 and in the Controlled Drug Disposition Record.

Skin test antigens are administered intradermally. A maximum of 8 injections may be given per animal and a maximum of 0.1 mL administered per site.

Injections sites are identified by marking them with indelible ink. The distal portion of the shoulder blades is used as a landmark to delineate the upper boundary of the injection area.

A line is then drawn down the center of the animal's back. Four more lines are drawn down the animal's back, perpendicular to the centerline and 30-40 mm apart (as shown below).



Skin tests are performed in the delineated areas on each animal and all appropriate information is recorded on DP 0018F1. On sites 1, 3, 5 and 7, dilutions 1 through 4 of LRS lot are injected. On sites 2, 4, 6, and 8, Dilutions 1 through 4 of LTSTA lot are injected. The volume of each injection is 0.1 mL.

3.2 Reading the Test

At 24±3 hours post skin test application, the edge of the longest and orthogonal axis of Induration is marked with dots or lines indicating the border of the reaction for each axis. Each axis is measured with a ruler to the nearest whole mm using the marks as guides. Reactions for each test site are recorded on DP 0018F1 (Appendix). The average of the longest diameter and the perpendicular (orthogonal) diameter is recorded as the skin test reaction size. A tabular format for recording the results (reactions) of each relative potency test is shown in Table 3.

Table 3. Relative Potency Test Results

lot	dil	GP1	GP2	GP3	GP4	Average
ref	1					
ref	0.5					
ref	0.25					
ref	0.125					
test	1					
test	0.5					
test	0.25					
test	0.125					

The average reaction of the four guinea pigs corresponding to each dilution is taken as the response variable. The dilution is the independent variable. These two variables, dilution and average reaction, are used to develop the relative potency test method.

4.0 Relative Potency Calculations Using a Linear Regression Model

A production lot is tested in parallel with the LRS using the four dilutions as explained above. The results of RP test are recorded in table 1. The dilutions (factors) are converted to logarithms to the base 10, that is,

X_1 = log (dil 1) = log (1) = 0.000

X_2 = log (dil 2) = log (0.5) = - 0.301

X_3 = log (dil 3) = log (0.25) = - 0.602

X_4 = log (dil 4) = log (0.125) = - 0.903

Assuming that Y_1, Y_2, Y_3, and Y_4 are the corresponding average reactions (see Table 3 above). Under the linear regression model, the lines for each lot will be of the type:

$Y = \alpha + \beta X$

where X = log (Dil) and Y = Response (average reaction, average over four guinea pigs, at that dilution);

β = Slope of the regression line and α = Y-Intercept.

The two regression lines can be expressed as: $Y = \alpha_r + \beta_r X$ and $Y = \alpha_t + \beta_t X$ for the reference and test lot, respectively.

The best fit linear regression lines $Y = A_r + B_r X$ and $Y = A_t + B_t X$ are calculated. Each slope is tested for significance from zero at alpha level of 0.1. That is, testing the hypothesis:

$H_0: \beta = 0$ vs. $H_A: \beta \neq 0$

The hypothesis must be rejected for both lots at alpha level of 0.1, i.e., the calculated p-value must be less than 0.1. This shows a significant linear relationship between log (dil) and response Y. Failing to reject the slope is zero for either one or both lots, the relative potency test is invalid and results are discarded.

Next, the two slopes are tested for equality at alpha level of 0.01. That is, testing the hypothesis:

$H_0: \beta_r = \beta_t$ vs. $H_A \beta_r \neq \beta_t$

If equality is not rejected, i.e. the calculated p-value is ≥ 0.01, the two regression lines are considered parallel. Calculating the common slope B and the two new intercepts A_r and A_t. The fitted parallel lines would be:

$Y = A_r + BX$ and $Y = A_t + BX$

The horizontal distance between the two parallel lines is the logarithm of the relative potency of the LtSTA test lot relative to the reference lot LRS. The log (RP) is given by the formula:

$\text{Log (RP)} = (A_t - A_r)/B$ and $RP = 10^{\log(RP)}$

The potency of the test lot is obtained by multiplying the RP by the potency of the LRS lot. Since the assigned potency of LRS is 30 μg per 0.1 mL, the potency of the production lot = 30 * calculated RP in μg per 0.1 mL.

5.0 DEVELOPMENT OF POTENCY LIMITS

5.1 Potency Limits for Release of a LTSTA Production Lot

To obtain 95% confidence limits for the release of LTSTA production lots, the variability of the potency obtained from the relative potency assay must be estimated. To estimate the variability of potency between lots, three production lots will be manufactured for testing in parallel with the LRS using the RPTM described above. A total of seven independent relative potency tests will be performed on each production lot, i.e., dilutions of lots, animals, etc will all be done separately within each relative potency assay.

In addition, the LRS will be tested against itself independently seven times. For testing the LRS against itself, two samples of LRS will be taken. One sample will be designated as the reference lot and the other sample as the test lot. Designation will be made before starting the seven independent relative potency tests and will be maintained consistently throughout.

Validation Protocol for a Relative Potency Test Method Used to Evaluate the Potency of *Leishmania tropica* Skin Test Antigen (LtSTA) with Respect to a LtSTA Internal Reference Standard (LRS)

BB-IND 11822

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Allermed Laboratories, Inc.
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September 10, 2008

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Validation Protocol for a Relative Potency Test Method
Used to Evaluate the Potency of *Leishmania tropica* Skin Test Antigen (LtSTA)
with Respect to a LtSTA Internal Reference Standard (LRS)

1.0 OBJECTIVE

The purpose of this protocol is to validate a Relative Potency Test Method (RPTM) procedure for evaluating the potency of *Leishmania tropica* Skin Test Antigen (LtSTA) lots with respect to a LtSTA Internal Reference Standard (LRS) and to demonstrate that when the procedure is executed as outlined, satisfactory analytical performance will be met. If the results meet the requirements defined below, the RPTM procedure for LtSTA will be considered validated.

2.0 BACKGROUND

Allermed Laboratories has developed a procedure that results in the production of manufactured lots of LtSTA that are comparable to the LRS in potency. It has been established that an LRS lot prepared by Allermed has a potency of 30 μg per 0.1 mL. Relative potency tests are reproducible and robust and are suitable for evaluating consistency of potency across manufactured lots of LtSTA. The side by side comparison of potency values of a manufactured lot with corresponding values of the LRS minimizes the variability of the test due to variation introduced by the technician, test animals, dose, and other factors. Because the effects of such variation are exerted on both articles being tested (manufactured lot and LRS), their impact on the results of the test is minimized, if not eliminated.

3.0 REGULATORY COMPLIANCE

This study will be conducted according to Allermed Laboratories Standard Operating Procedures and current Good Manufacturing Practices as outlined in the 21 CFR Parts 210 and 211 (1). This protocol meets the applicable guidance identified by the United States Pharmacopoeia 24, General Chapter <1225> (2) and the International Conference on Harmonisation (ICH) Guidelines Q2A (3) and Q2B (4) in terms of the analytical performance characteristics identified in the protocol below.

4.0 MATERIAL/EQUIPMENT/SAMPLES/STANDARDS

Samples

LRS (the internal positive control) and two LtSTA manufactured lots (LtSTA01 and LtSTA02), will be used in this protocol. The lots will be used at 100% nominal concentration and used to validate the precision of the relative potency test method procedure. The LRS lot will be used as the reference at 30.0 μg per 0.1 mL.

Once the 95% confidence interval (CI) limits for the potency of a LtSTA manufactured lot are established, five samples of LtSTA with nominal concentrations will be made to validate linearity and the range of the RPTM procedure.

Sample 1 will have a concentration below the 80% of CI lower limit and Sample 5 will have a concentration slightly above the 120% of the CI upper limit. The other three samples will be equally spaced in between the two concentrations. There is no internationally accepted second method for determining an independent LtSTA concentration. The LRS and LtSTA test lots will be run at four dilutions, 1:1, 1:2, 1:4, and 1:8 of the nominal concentrations of the LRS and the LtSTA test lots. All other materials/equipment/guinea pig sensitization agents/animals necessary will be documented in laboratory records maintained by Allermed.

5.0 CALCULATION/VALIDITY REQUIREMENTS/DATA RECORDS

5.1 Estimating relative potency with a parallel line method

Calculation of the relative potency of an LtSTA test lot is based upon parallel-line methodology. This model assumes that the test lot acts as a dilution of the reference lot (LRS). Mathematically this can be expressed as:

$$D_t = D_r/RP$$

Where:

RP = relative potency factor

D_t = dose of the test lot

D_r = dose of the reference lot

(Note: r denotes reference and t denotes test)

If the assay response, Y is modeled as a linear function of log (dose), it can be written:

$$Y_r = \alpha + \beta \log (D_r)$$

$$Y_t = \alpha + \beta \log (D_t)$$

Where:

β = slope

α = Y-intercept

There are two validity criteria for a parallel line relative potency assay:

(1) Slopes of reference line and test line are significantly different from zero at alpha level of 0.1.

(2) Slope of the reference line cannot be significantly different from that of the test line at alpha level of 0.01.

The common slope is defined as β. If this is placed into the equations above, it can be shown that:

$$\text{Log (RP)} = (\alpha_t - \alpha_r)/ \beta$$

The $(\alpha_t - \alpha_r)/ \beta$ is the horizontal distance between the reference and test log(dose)-response lines. The calculation of LRS potency as outlined in the attached RP test method SOP uses A and B as estimates of the parameters α and β, respectively. The LtSTA log(dose)-response line is generated by four doses, 1:1, 1:2, 1:4, and 1:8 for test and reference lot each and is modeled using a linear regression model Y on X, where Y is response and X is log(dose).

5.2 Data Records

Completed forms, including those for invalid tests, will be permanently affixed to the validation notebook. Records will be maintained in accordance with Allermed Laboratories record maintenance policies.

6.0 SAFETY PRECAUTIONS

Normal laboratory safety precautions including the handling of biohazardous materials and waste will be followed, including any described in the Material Safety Data Sheets or appropriate Allermed Laboratories SOP dealing with biohazardous materials.

7.0 ANALYTICAL PERFORMANCE CHARACTERISTICS

7.1 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision will be considered at two levels: Repeatability and Intermediate Precision.

7.1.1 Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed Intra-Assay Precision.

Method: Two LtSTA test lots, LtSTA01 and LtSTA02, will be analyzed. On each lot three replicate tests of relative potency will be performed. If possible, all six tests will start on the same day or as close together as possible.

Data: Required data sheets – one per test: LtSTA Dilution Record, LtSTA Potency Test Record, etc. Final results will be presented in a table (Table 2) containing mean, standard deviation (SD) and percent relative standard deviation (%RSD) of three potency values of each lot.

Table 2. Potency assay results for precision (repeatability)

Lot	Sample 1	Sample 2	Sample 3	Mean	SD	%RSD
LtSTA01						
LtSTA02						

Acceptance Criteria: %RSD = (SD/ Mean)*100 within a lot be ≤ 50%.

7.1.2 Intermediate Precision/Ruggedness

Intermediate precision expresses between run variations. The ruggedness of the test is determined by introducing as much typical variability as possible during the validation. Therefore, tests will be run during different weeks with three different analysts.

Method: One LtSTA test lot, LtSTA01, will be analyzed. Three intermediate precision tests will be run, one test per analyst, i.e., test 1 by analyst 1 on week1, test 2 by analyst 2 on week 2 and test 3 by analyst 3 on week 3. Each test will have a unique combination of analyst and test date. Each test will have different sensitized guinea pigs.

Data: Required data sheets per test, LtSTA Dilution Record, LtSTA Potency Test Record, etc. Final results will be presented in a table (Table 3) containing mean, standard deviation (SD) and percent relative standard deviation (%RSD) of the three potency values.

Table 3. Potency assay results for intermediate precision/ruggedness

Test	Analyst	Lot	Potency Value
Intermediate Precision 1		LtSTA01	
Intermediate Precision 2		LtSTA01	
Intermediate Precision 3		LtSTA01	
		N	3
		Mean	
		SD	
		%RSD	

Acceptance Criteria: %RSD within a lot must be ≤ 50%.

7.2 Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample which has suitable precision, accuracy and linearity. Precision has been characterized above. This characterization, therefore, concentrates upon accuracy throughout the proposed test method range.

Method: Five samples from S1 to S5 with different nominal concentrations of LtSTA will be formed as stated above in table 1. The nominal concentrations of these samples are given in the table. Each sample will be tested only once to obtain the potency by the RPTM.

Data: Required data sheets per test, LtSTA Dilution Record, LtSTA Potency Test Record, etc. The final results will be presented in a table (Table 4) containing theoretical potency and calculated potency values by Sample.

Table 4. Potency assay results for range

Sample	Theoretical Potency	Calculated Potency
Sample S1		
Sample S2		
Sample S3		
Sample S4		
Sample S5		

The calculated potency values (Y-axis) for each test result will be plotted against the theoretical potency values (X-axis) and analyzed with a best-fit linear regression.

Acceptance Criteria: The slope of the calculated potency vs. theoretical potency line must be between 0.50 and 2.0, inclusive.

7.3 Linearity

Linearity of the method is the ability of the test method to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the LtSTA concentration in samples within a given range.

Method: The calculated potencies of the five samples in the Range will be plotted against their nominal concentrations (theoretical potencies). The best fit linear regression line

$$Y = A + BX$$

will be obtained and plotted on the graph, where Y is the response (calculated potency), X is the expected or theoretical potency, B is slope, and A is Y-intercept.

Acceptance Criteria: The R^2, slope B, and Y-intercept will be reported. R^2 must be ≥ 0.85; slope B must be between 0.50 and 2.0, inclusive.

7.4 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between value, which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

Since there is no accepted LtSTA international reference standard, the accuracy of this assay is inferred by analyzing the range results for % recovery of the nominal concentration test samples compared to LRS. Data will be presented in a table (Table 5) with columns of theoretical potency, calculated potency, and percent recovery.

Percent recovery = (Calculated potency/Theoretical potency) * 100

Table 5. Potency Assay Results for Accuracy

Sample	Theoretical Potency	Calculated Potency	% Recovery
Sample S1			
Sample S2			
Sample S3			
Sample S4			
Sample S5			

Acceptance Criteria: Recovery ranges from 50% to 150%, inclusive for all five samples.

8.0 REPORT

Upon completion of the protocol, a draft report and the final test method will be prepared, evaluated and reviewed. The final report will contain all relevant protocols, data sheets and results as described above in the document. A final report in the standard format of Allermed Laboratories and a Method SOP will then be issued.

9.0 MAINTENANCE OF RAW DATA

Once data sheets are completed they will be maintained in the appropriate validation notebook. Raw data, or true copies, will be available at Allermed Laboratories to facilitate auditing the study. When the final report is completed all raw data, and a copy of the final report will be retained in the archives of Allermed Laboratories per their standard policy.

10.0 REFERENCES

1. 21 Code of Federal Regulations (CFR), Parts 210 and 211
2. Current United States Pharmacopoeia, General Chapter <1225>
3. International Conference on Harmonization: Guideline for Industry: Text on Validation of Analytical Procedures, ICH-Q2A, 1995
4. International Conference on Harmonization: Guideline for Industry: Q2B Validation of Analytical Procedures: Methodology, ICH-Q2B, November 1996

11.0 ATTACHMENTS

1. Attachment A - RP Test Method SOP

Attachment 5

Identity Test Method and Validation Protocol.

Identity Test Method (RPTM)
for Evaluating the Identity of *Leishmania tropica* Skin Test Antigen (LtSTA) Lots with Respect to a LtSTA Internal Reference Standard (LRS)

BB-IND 11822

Sponsor
Allermed Laboratories, Inc.
7203 Convoy Court
San Diego, CA 92111

September 10, 2008

STANDARD OPERATING PROCEDURE: ***Leishmania tropica* Identity Test** 176

I. PURPOSE

This document describes the procedure for identifying *Leishmania tropica* via an Enzyme linked Immunosorbant Assay (ELISA) from other extracts produced at Allermed laboratories

II. SCOPE

This document applies to the testing of *Leishmania tropica* at 30 μg/mL (the concentration used for sensitivity testing) in an indirect ELISA.

III. RESPONSIBILITIES

QC employees of Allermed Laboratories are responsible for following this procedure.

IV. REFERENCES/RELATED DOCUMENTS

DP 1029	Production of *Leishmania tropica* Positive Control
SOP 916-000	Assay for total Protein by Ninhydirn
SOP FORM 944-102F1	*Leishmania tropica* Identity Test Result Form
EP 010	Pooling Rabbit Anti-*Leishmania tropica* Immune Sera

V. DEFINITIONS

ABTS- 2, 2'-Azino-bis (3-ethylbenzothiazoline-6 sulfonic acid)
BSA-Bovine serum albumin
HRP-Horse radish peroxidase
ELISA-Enzyme linked Immunosorbent assay
PBS-Phosphate buffered saline
SDS-Sodium dodecyl sulfate
Wash Solution – 0.17% Brij 35 in PBS
Blocking Solution-1% BSA in PBS
ABTS Stop Solution- 1% SDS in DI water
DI-Deionized water

VI. MATERIALS, SUPPLIES, AND EQUIPMENT

Microtiter plates (Coring-Costar 3590)
Plate sealers (Fisher # 08-408-240 or equivalent)
Reservoirs (VWR # 89031-328 or equivalent)
Multi channel pipetter
Automatic pipetter
Pipette tips
Microcentrifuge tubes (VWR # 89000-028)
Leishmania tropica positive control (Allermed Laboratories-prepared according to **DP 1029**)
Rabbit anti-*Leishmania tropic* serum (Pooled rabbit serum immunized with *Leishmania tropica-Greer Laboratories)*
Goat anti-rabbit horseradish peroxidase labeled IgG (KPL Cat. # 074-1506)
Candin (Allermed Laboratories)
Chaetomium globbosum allergenic extract 1:10 w/v (Allermed Laboratories)
Coccidioidin SD (Allermed Laboratories)
ABTS (KPL Cat. # 50-66-18)
ABTS Stop Solution (KPL # 50-85-01)
BSA (KPL # 50-61-00)
Brij 35 (VWR # 3844-1 or equivalent)
10 X PBS (KD Medical # RGF-3210)
Microtiter plate reader (Molecular Devices or equivalent) Graduated cylinders Paper towels
Magnetic stirrer Beakers 10 mL pipets Gloves Stir bars

VII. SAFETY REQUIREMENTS

Don lab coats, gloves and safety goggles when working with these reagents.

VIII. PROCEDURE

A. Sample Preparation

Determine the protein concentration of all samples (test, controls and standard) following **SOP 916-000**.

B. Reagent Preparation (Prepare the day of use)

1. Thaw the rabbit anti-Leishmania tropicas serum in the refrigerator overnight (use within 5 days). Prepare 10 mL of the primary antibody per plate by diluting 1:5000 in PBS (2µL of serum in 10 mL of 1X PBS).
2. Prepare 100 mL of 1X PBS (10 mL of 10X PBS in 90 mL of DI water).
3. Prepare 500 mL of wash buffer (5 mL of 10X PBS in 495 mL of DI water + 850µL of Brij 114)
4. Prepare 20 mL of blocking buffer per plate (2 mL of the 10% BSA solution in 18 mL of DI water).
5. Prepare 10 mL of the secondary antibody per plate by diluting 1:2000 in blocking buffer (5µL of the goat anti-rabbit IgG HRP in 10 mL of blocking buffer).
6. Prepare 10 mL of stop solution per plate (2 mL of the ABTS stop solution in 8 mL of DI water).

C. ELISA Assay

1. Antigen Coating Procedure (see template)
   Dilute the following samples (positive control, Candin, *Chaetomium globbosum* and test samples) to 30 ug/mL with PBS. Use the Coccidioidin SD undiluted.
   (need 500 µL of each per plate).
   a. *L. tropica* positive control- Add 100 µL/well to Row 1 A-D.
   b. Negative Controls:
      1. Candin- Add 100 µL/well to Row 2A-D.
      2. *Chaetomium globbosum*- Add 100 µL/well to Row 3 A-D.
      3. *Coccidioides immitis* -Add 100 µL/well to Row 7 A-D
   c. Reagent controls (non-specific background):
      1. Non-specific plate binding control -Add 100 µL/well of PBS to Row 4 A-D.
      2. Non-specific antiserum binding control- Add 100 µL/well of PBS to Row 6 A-D.
      3. Non-specific horseradish peroxidase binding control- Add 100 µL/well of PBS to Row 6 E-H.
   d. Test sample(s): Add 100 µL/well of Product to Row 5 A-H. Use columns 8-12 for additional samples.
   e. Tap to dislodge air bubbles.
   f. Cover with plate sealer.
   g. Incubate at room temperature for 30 minutes.
2. Blocking Procedure
   a. Dump the coating solution into the sink and blot plate on a paper towel to remove residual fluid.
   b. Add 200 µL of blocking solution to all wells. Gently tap to dislodge any air bubbles.
   c. Cover with plate sealer.
   d. Incubate at room temperature for a minimum of 30 minutes.
3. 1st Wash Step (Use an automatic plate washer or perform manually)
   a. Aspirate off solution and add ~ 400 µL of B114 per well.
   b. Repeat two more times.
   c. Blot plate on a paper towel to remove any residual fluid.
4. Addition of Primary Antibody
   a. Dilute rabbit serum 1:5000 with PBS.
   b. Add 100 µL to each well, except Row 6 E-H (HRP Control).
   c. Add 100 µL of blocking buffer to Row 6 E-H.
   d. Gently tap plate to dislodge any air bubbles.
   e. Cover with plate sealer.
   f. Incubate at room temperature for 30 minutes.
5. 2nd Wash Step (Use an automatic plate washer or perform manually)
   a. Dump the antibody solution into the sink.
   b. Aspirate off solution and add ~400 µL of B114 per well.
   c. Repeat two more times.
   d. Blot plate on a paper towel to remove any residual fluid.

6. Addition of Secondary Antibody

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a. Dilute the goat anti-rabbit IgG HRP 1:2000 with blocking buffer.
b. Add 100 µL to each well, except Row 6 A-D (Serum Control).
c. Add 100 µL of blocking buffer to Row 6 A-D.
d. Gently tap plate to dislodge any air bubbles.
e. Cover with plate sealer.
f. Incubate at room temperature for 30 minutes.

7. 3rd Wash step (Use an automatic plate washer or perform manually)
   a. Aspirate off solution and add ~400 µL of B114 per well.
   b. Repeat two more times.
   c. Blot plate on paper towel to remove any residual fluid.
8. Addition of Substrate and Stop Solution
   a. Warm the ABTS substrate to room temperature.
   b. Add 100 µL/well of substrate and incubate for approximately 60 minutes at room temperature.
   c. Add 100 µL/well of stop solution. Gently tap plate to dislodge any air bubbles.
   d. Read plates at 405 nm on a spectrophotometer.

Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	*L. tropica* Positive Control	Candin	*Chaetomium globbosum*	Non- specific plate binding Control	Test Sample 1	Non-specific anti-serum binding Control	Coccidioidin SD	Test Sample 2	Test Sample 3	Test Sample 4	Test Sample 5	Test Sample 6
B	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E						Non-specific HRP binding Control						
F						↓						
G						↓						
H						↓						

IX. RESULTS

A. Data Analysis

Record the required information on **SOP Form 944-102F1**.

B. Acceptance Criteria

1. For the assay to be valid:
   a. The average absorbance value for the background controls shall be ≤ 0.100.
   b. The average absorbance value for the negative controls shall be ≤ 0.300.
   c. The average absorbance value for the SIS standard and the test sample shall be ≥ 2x the highest negative control absorbance.
2. If any of the above parameters are not met, the assay is invalid and shall be repeated.

Validation Protocol for an Identity Test Method Used to Evaluate the Identity of *Leishmania tropica* Skin Test Antigen (LtSTA) with Respect to a LtSTA Internal Reference Standard (LRS)

BB-IND 11822

Sponsor
Allermed Laboratories, Inc.
7203 Convoy Court
San Diego, CA 92111

September 10, 2008

I. Introduction

The purpose of this validation is to demonstrate the specificity and precision of the LtSTA Identity Test. Precision is evaluated at two levels: Repeatability or Intra-assay Precision, and Intermediate Precision or Reproducibility/Ruggedness (Inter-assay Precision).

II. Specificity of LtSTA Identity Test

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. For identification tests, it is necessary to ensure the identity of the analyte.

In this validation, the ELISA assay will be used to compare the LtSTA against positive and negative controls. The rabbit anti-LtSTA serum that will be used in the assay was obtained by immunizing rabbits with freeze-thaw ruptures promastigotes of *L. tropica* (WR# 1063-CIA).

A. Method

The LtSTA Identity Test will be performed using an indirect ELISA procedure with rabbit anti-*L. tropica* serum as the primary antibody and goat anti-rabbit IgG HRP as the secondary antibody. The reaction is visualized with ABTS and read spectrophotometrically at 405 nm. The positive control (LRS) and the production lot of LtSTA produce a dark color in comparison to the negative controls. The background controls show no sign of color development.

B. Acceptance Criteria

1. The average absorbance (OD) values for negative controls shall be < 0.300.
2. The average absorbance values for LRS and LtSTA lots (test samples) shall be $\geq 2X$ (the highest control absorbance value among background and negative controls).
3. The average absorbance of the background controls shall be ≤ 0.100.
4. All assays performed shall meet acceptance criteria.

III. Intra-Assay Precision of LtSTA Identity Test

A. Method

The repeatability (intra-assay precision) of the test will be addressed by having one technician perform three assays (three plates) consecutively in a single day with three different lots of LtSTA.

B. Acceptance Criteria (see II.B. above)

IV. Inter-Assay Precision of LtSTA Identity Test

A. Method

The inter-assay precision of the test will be addressed by having three technicians perform three assays (three plates) over three days with one lot of LtSTA. Each plate will contain negative controls, background controls, LRS and one test sample of LtSTA.

B. Acceptance Criteria (see II.B. above)